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*Attorneys for Plaintiff  
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**UNITED STATES DISTRICT COURT  
DISTRICT OF NEW JERSEY**

**BIOMARIN PHARMACEUTICAL INC.  
and MERCK & CIE,**

**Plaintiffs,**

**v.**

**DR. REDDY'S LABORATORIES, INC.  
and DR. REDDY'S LABORATORIES,  
LTD.,**

**Defendants.**

**Civil Action No. \_\_\_\_\_**

**COMPLAINT FOR  
PATENT INFRINGEMENT**

**(Filed Electronically)**

Plaintiffs BioMarin Pharmaceutical Inc. ("BioMarin") and Merck & Cie ("Merck")  
(collectively, "Plaintiffs"), by their undersigned attorneys, for their complaint against Dr.  
Reddy's Laboratories, Inc. and Dr. Reddy's Laboratories, Ltd. (collectively, "DRL"), allege as  
follows:

### **NATURE OF THE ACTION**

1. This is an action for patent infringement under the patent laws of the United States, Title 35 of the United States Code, arising from DRL's filing of a purported Abbreviated New Drug Application ("ANDA") with the United States Food and Drug Administration ("FDA") seeking approval to commercially manufacture and market a generic version of the pharmaceutical drug product Kuvan<sup>®</sup> prior to the expiration of U.S. Patent Nos. 7,566,462 ("the '462 patent"), 7,566,714 ("the '714 patent"), 7,612,073 ("the '073 patent"), 8,003,126 ("the '126 patent"), 8,067,416 ("the '416 patent"), RE43,797 ("the '797 patent"), and 8,318,745 ("the '745 patent") (collectively, the "patents-in-suit").

### **THE PARTIES**

2. Plaintiff BioMarin is a corporation organized and existing under the laws of the State of Delaware, having a principal place of business at 770 Lindero Street, San Rafael, California 94901.

3. Plaintiff Merck is a Swiss corporation having a principal place of business at WeissHausmatte, 6469 Altdorf, Switzerland.

4. Upon information and belief, Dr. Reddy's Laboratories, Inc. is a corporation incorporated under the laws of the State of New Jersey, having a principal place of business at 107 College Road East, Princeton, New Jersey 08540. Upon information and belief, Dr. Reddy's Laboratories, Inc. is in the business of, among other things, marketing and selling generic versions of branded pharmaceutical products, which it distributes in New Jersey and throughout the United States.

5. Upon information and belief, Dr. Reddy's Laboratories, Ltd. is a company organized under the laws of India, having a principal place of business at 8-2-337, Road No. 3, Banjara Hills, Hyderabad, 500 034, India. Upon information and belief, Dr. Reddy's

Laboratories, Ltd. is in the business of making and selling generic pharmaceutical products, which it distributes in New Jersey and throughout the United States through at least Dr. Reddy's Laboratories, Inc.

6. Upon information and belief, Dr. Reddy's Laboratories, Inc. is a subsidiary of Dr. Reddy's Laboratories, Ltd.

7. Upon information and belief, Dr. Reddy's Laboratories, Inc. is the exclusive agent in North America for Dr. Reddy's Laboratories, Ltd.

### **JURISDICTION AND VENUE**

8. Subject matter jurisdiction over this action is premised on 28 U.S.C. §§ 1331 and 1338(a).

9. This Court has personal jurisdiction over DRL by virtue of, *inter alia*, DRL having a presence in New Jersey, DRL having conducted business in New Jersey, DRL having availed itself of the rights and benefits of New Jersey law, DRL purposefully availing itself of the privilege of conducting business in New Jersey, DRL having previously consented to personal jurisdiction in this Court, and DRL having engaged in systematic and continuous contacts with the State of New Jersey.

10. DRL has stipulated and/or consented to personal jurisdiction before this Court in other patent cases including: *Janssen Pharmaceutica N.V. v. Dr. Reddy's Labs., Ltd.*, 03-6185 D.N.J. (JWB); *Teva Pharm. Indus. v. Dr. Reddy's Labs., Ltd.*, 07-2894 D.N.J. (GEB) (JJH); *Hoffmann La Roche Inc. v. Dr. Reddy's Labs., Ltd.*, 07-4516 D.N.J. (SRC) (CCC); *Astrazeneca UK Ltd. v. Dr. Reddy's Labs., Ltd.*, 08-3237 D.N.J. (MLC) (TJB); *Albany Molecular Research, Inc. v. Dr. Reddy's Labs., Ltd.*, 09-4638 D.N.J. (JAG) (MCA); *The Meds. Co. v. Dr. Reddy's Labs., Ltd.*, 11-2456 D.N.J. (PGS) (DEA); *Helsinn Healthcare S.A. v. Dr. Reddy's Labs., Ltd.*, 12-2867 D.N.J. (MLC) (DEA); and *Astrazeneca AB v. Dr. Reddy's Labs., Ltd.*, 13-91 D.N.J.

(JAP) (TJB). Furthermore, DRL asserted counterclaims in a majority of these cases, thus availing itself of the benefits and protections of the laws of New Jersey and its court system.

11. Venue is proper in this judicial district pursuant to 28 U.S.C. §§ 1391 and 1400(b).

### **THE PATENTS-IN-SUIT**

12. On July 28, 2009, the United States Patent and Trademark Office (“USPTO”) duly and lawfully issued the ’462 patent, entitled “Stable Tablet Formulation,” to BioMarin as assignee of the inventors Steven Jungles, Mark A. Henderson, Victoria Sluzky, and Robert Baffi. A copy of the ’462 patent is attached hereto as Exhibit A.

13. BioMarin is the owner of all right, title, and interest in the ’462 patent.

14. On July 28, 2009, the USPTO duly and lawfully issued the ’714 patent, entitled “Methods and Compositions for the Treatment of Metabolic Disorders,” to BioMarin and Merck Eprova AG as assignees of inventors Daniel I. Oppenheimer, Emil D. Kakkis, Frederic D. Price, Alejandro Dorenbaum, Rudolf Moser, Viola Groehn, Thomas Egger, and Fritz Blatter. Merck Eprova AG subsequently assigned all of its interest in the ’714 patent to BioMarin. A copy of the ’714 patent is attached hereto as Exhibit B.

15. BioMarin is the owner of all right, title, and interest in the ’714 patent.

16. On November 3, 2009, the USPTO duly and lawfully issued the ’073 patent, entitled “Methods of Administering Tetrahydrobiopterin, Associated Compositions, and Methods of Measuring,” to BioMarin as assignee of inventors Daniel I. Oppenheimer and Alejandro Dorenbaum. A copy of the ’073 patent is attached hereto as Exhibit C.

17. BioMarin is the owner of all right, title, and interest in the ’073 patent.

18. On August 23, 2011, the USPTO duly and lawfully issued the ’126 patent, entitled “Stable Table Formulation,” to BioMarin as assignee of inventors Steven Jungles, Mark

Henderson, Victoria Sluzky, and Robert Baffi. A copy of the '126 patent is attached hereto as Exhibit D.

19. BioMarin is the owner of all right, title, and interest in the '126 patent.

20. On November 29, 2011, the USPTO duly and lawfully issued the '416 patent, entitled "Methods and Compositions for the Treatment of Metabolic Disorders," to BioMarin and Merck Eprova AG as assignees of inventors Daniel I. Oppenheimer, Emil D. Kakkis, Frederic D. Price, Alejandro Dorenbaum, Rudolf Moser, Viola Groehn, Thomas Egger, and Fritz Blatter. Merck Eprova AG subsequently assigned all of its interest in the '416 patent to BioMarin. A copy of the '416 patent is attached hereto as Exhibit E.

21. BioMarin is the owner of all right, title, and interest in the '416 patent.

22. On November 6, 2012, the USPTO duly and lawfully issued the '797 patent, entitled "Methods of Administering Tetrahydrobiopterin," to BioMarin as assignee of inventors Daniel I. Oppenheimer, Alejandro Dorenbaum, and Augustus O. Okhamafe. The '797 patent is a reissue of U.S. Patent No. 7,947,681. A copy of the '797 patent is attached hereto as Exhibit F.

23. BioMarin is the owner of all right, title, and interest in the '797 patent.

24. On November 27, 2012, the USPTO duly and lawfully issued the '745 patent, entitled "Crystalline Forms of (6R)-L-Erythro-Tetrahydrobiopterin Dihydrochloride," to Merck Eprova AG as assignee of inventors Rudolf Moser, Viola Groehn, Thomas Egger, and Fritz Blatter. Subsequently, Merck Eprova AG assigned the '745 patent to Merck & Cie KG, which then changed its name to Merck & Cie. A copy of the '745 patent is attached hereto as Exhibit G.

25. Merck is the owner of all right, title, and interest in the '745 patent. BioMarin is the exclusive licensee of the '745 patent.

**The Kuvan<sup>®</sup> Drug Product**

26. BioMarin holds approved New Drug Application (“NDA”) No. 022181 for oral tablets containing 100 mg of sapropterin dihydrochloride, sold under the trade name Kuvan<sup>®</sup>.

27. Pursuant to 21 U.S.C. § 355(b)(1) and attendant FDA regulations, the patents-in-suit are listed in the FDA publication, “Approved Drug Products with Therapeutic Equivalence Evaluations” (the “Orange Book”), with respect to Kuvan<sup>®</sup>.

**DRL’s FDA Submission**

28. Upon information and belief, DRL submitted to the FDA documentation purporting to constitute an ANDA pursuant to 21 U.S.C. § 355(j), seeking approval to commercially manufacture, use, and market a generic version of the pharmaceutical drug product Kuvan<sup>®</sup> in the form of oral tablets containing 100 mg of sapropterin dihydrochloride (“DRL’s Generic Product”), prior to the expiration of the ’462, ’714, ’073, ’126, ’416, ’797, and ’745 patents.

29. DRL’s purported ANDA relies upon the Kuvan<sup>®</sup> NDA and, according to DRL, contains the required data with respect to the bioavailability or bioequivalence of DRL’s Generic Product to Kuvan<sup>®</sup>.

30. BioMarin and Merck received letters from DRL, dated October 3, 2014, with attached memoranda (collectively, “DRL’s Notification”), stating that DRL included certifications in its FDA submission, pursuant to 21 U.S.C. § 355(j)(2)(A)(vii)(IV), that the patents-in-suit are invalid, unenforceable, and/or will not be infringed by the commercial manufacture, use, or sale of DRL’s Generic Product (the “Paragraph IV certification”). Thus, DRL is seeking approval of its proposed Generic Product prior to the expiration of the patents-in-suit. Plaintiffs are filing this complaint within the 45-day interval from receipt of DRL’s

Notification, pursuant to 21 U.S.C. § 355(c)(3)(C). Plaintiffs reserve all rights to challenge the sufficiency of DRL's purported ANDA and Paragraph IV certification.

**COUNT ONE: INFRINGEMENT OF THE '462 PATENT**

31. Plaintiffs repeat and reallege the allegations of paragraphs 1–30 as though fully set forth herein.

32. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '462 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

33. Unless enjoined by this Court, upon FDA approval, DRL will infringe the '462 patent under 35 U.S.C. § 271(a) by making, using, offering to sell, importing, and/or selling DRL's Generic Product in the United States.

34. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '462 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '462 patent and knowledge that its acts are encouraging infringement.

35. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '462 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '462 patent and that there is no substantial non-infringing use for DRL's Generic Product.

36. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '462 patent, warrant a finding that this case is an

exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

37. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '462 patent is not enjoined.

38. Plaintiffs do not have an adequate remedy at law.

**COUNT TWO: INFRINGEMENT OF THE '714 PATENT**

39. Plaintiffs repeat and reallege the allegations of paragraphs 1–38 as though fully set forth herein.

40. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '714 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

41. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '714 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '714 patent and knowledge that its acts are encouraging infringement.

42. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '714 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '714 patent and that there is no substantial non-infringing use for DRL's Generic Product.

43. DRL does not contest infringement of claims 1-28 and 43-46 of the '714 patent in DRL's Notification. If DRL had a factual or legal basis to contest infringement of claims 1-28 and 43-46 of the '714 patent, it was required by applicable regulations to state such basis in



DRL's Notification. *See* 21 CFR 314.52 (requiring paragraph IV notice letter to include a detailed, claim-by-claim analysis of its bases for claiming non-infringement or invalidity of any patent that is listed in the Orange Book in conjunction with the reference listed drug and as to which the applicant has submitted a paragraph IV certification).

44. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '714 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

45. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '714 patent is not enjoined.

46. Plaintiffs do not have an adequate remedy at law.

**COUNT THREE: INFRINGEMENT OF THE '073 PATENT**

47. Plaintiffs repeat and reallege the allegations of paragraphs 1–46 as though fully set forth herein.

48. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '073 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

49. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '073 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '073 patent and knowledge that its acts are encouraging infringement.

50. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '073 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had

and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '073 patent and that there is no substantial non-infringing use for DRL's Generic Product.

51. DRL does not contest infringement of claims 1, 2, 5, 6, and 8 of the '073 patent in DRL's Notification. If DRL had a factual or legal basis to contest infringement of claims 1, 2, 5, 6, and 8 of the '073 patent, it was required by applicable regulations to state such basis in DRL's Notification. *See* 21 CFR 314.52 (requiring paragraph IV notice letter to include a detailed, claim-by-claim analysis of its bases for claiming non-infringement or invalidity of any patent that is listed in the Orange Book in conjunction with the reference listed drug and as to which the applicant has submitted a paragraph IV certification).

52. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '073 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

53. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '073 patent is not enjoined.

54. Plaintiffs do not have an adequate remedy at law.

#### **COUNT FOUR: INFRINGEMENT OF THE '126 PATENT**

55. Plaintiffs repeat and reallege the allegations of paragraphs 1–54 as though fully set forth herein.

56. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '126 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

57. Unless enjoined by this Court, upon FDA approval, DRL will infringe the '126 patent under 35 U.S.C. § 271(a) by making, using, offering to sell, importing, and/or selling DRL's Generic Product in the United States.

58. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '126 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '126 patent and knowledge that its acts are encouraging infringement.

59. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '126 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '126 patent and that there is no substantial non-infringing use for DRL's Generic Product.

60. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '126 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

61. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '126 patent is not enjoined.

62. Plaintiffs do not have an adequate remedy at law.

**COUNT FIVE: INFRINGEMENT OF THE '416 PATENT**

63. Plaintiffs repeat and reallege the allegations of paragraphs 1–62 as though fully set forth herein.

64. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin

dihydrochloride prior to the expiration of the '416 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

65. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '416 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '416 patent and knowledge that its acts are encouraging infringement.

66. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '416 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '416 patent and that there is no substantial non-infringing use for DRL's Generic Product.

67. DRL does not contest infringement of any claim of the '416 patent in DRL's Notification. If DRL had a factual or legal basis to contest infringement of any claim of the '416 patent, it was required by applicable regulations to state such basis in DRL's Notification. *See* 21 CFR 314.52 (requiring paragraph IV notice letter to include a detailed, claim-by-claim analysis of its bases for claiming non-infringement or invalidity of any patent that is listed in the Orange Book in conjunction with the reference listed drug and as to which the applicant has submitted a paragraph IV certification).

68. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '416 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

69. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '416 patent is not enjoined.

70. Plaintiffs do not have an adequate remedy at law.

**COUNT SIX: INFRINGEMENT OF THE '797 PATENT**

71. Plaintiffs repeat and reallege the allegations of paragraphs 1–70 as though fully set forth herein.

72. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '797 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

73. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '797 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '797 patent and knowledge that its acts are encouraging infringement.

74. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '797 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '797 patent and that there is no substantial non-infringing use for DRL's Generic Product.

75. DRL does not contest infringement of claims 1, 3-8, 10-14, and 18-27 of the '797 patent in DRL's Notification. If DRL had a factual or legal basis to contest infringement of claims 1, 3-8, 10-14, and 18-27 of the '797 patent, it was required by applicable regulations to state such basis in DRL's Notification. *See* 21 CFR 314.52 (requiring paragraph IV notice letter to include a detailed, claim-by-claim analysis of its bases for claiming non-infringement or

invalidity of any patent that is listed in the Orange Book in conjunction with the reference listed drug and as to which the applicant has submitted a paragraph IV certification).

76. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '797 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

77. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '797 patent is not enjoined.

78. Plaintiffs do not have an adequate remedy at law.

**COUNT SEVEN: INFRINGEMENT OF THE '745 PATENT**

79. Plaintiffs repeat and reallege the allegations of paragraphs 1–78 as though fully set forth herein.

80. Submission of an ANDA to obtain approval to engage in the commercial use, manufacture, sale, offer for sale, or importation of oral tablets containing 100 mg of sapropterin dihydrochloride prior to the expiration of the '745 patent constitutes infringement of one or more of the claims of that patent under 35 U.S.C. § 271(e)(2)(A).

81. Unless enjoined by this Court, upon FDA approval, DRL will infringe the '745 patent under 35 U.S.C. § 271(a) by making, using, offering to sell, importing, and/or selling DRL's Generic Product in the United States.

82. Unless enjoined by this Court, upon FDA approval, DRL will induce infringement of the '745 patent under 35 U.S.C. § 271(b). Upon information and belief, upon FDA approval, DRL will intentionally encourage acts of direct infringement with knowledge of the '745 patent and knowledge that its acts are encouraging infringement.

83. Unless enjoined by this Court, upon FDA approval, DRL will contributorily infringe the '745 patent under 35 U.S.C. § 271(c). Upon information and belief, DRL has had and continues to have knowledge that DRL's Generic Product is especially made or especially adapted for a use that infringes the '745 patent and that there is no substantial non-infringing use for DRL's Generic Product.

84. DRL's actions, including its reliance on the purported defenses and statements set forth in DRL's Notification regarding the '745 patent, warrant a finding that this case is an exceptional case pursuant to 35 U.S.C. § 285, and entitle Plaintiffs to recovery of their attorneys' fees and such other relief as this Court deems proper.

85. Plaintiffs will be substantially and irreparably harmed if DRL's infringement of the '745 patent is not enjoined

86. Plaintiffs do not have an adequate remedy at law.

#### **PRAYER FOR RELIEF**

WHEREFORE, Plaintiffs BioMarin and Merck pray for a Judgment in their favor and against DRL, and respectfully request the following relief:

A. A Judgment be entered that DRL has infringed the '462, '714, '073, '126, '416, '797, and '745 patents;

B. A Judgment pursuant to 35 U.S.C. § 271(e)(4)(B) preliminarily and permanently enjoining DRL, its officers, agents, servants, employees, and those persons in active concert or participation with any of them, from commercially manufacturing, using, offering to sell, or selling DRL's Generic Product within the United States, or importing DRL's Generic Product into the United States, prior to the expiration of the patents-in-suit;

C. A Judgment ordering that, pursuant to 35 U.S.C. § 271(e)(4)(A), the effective date of any approval of ANDA No. 207685 under § 505(j) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355(j)) shall not be any earlier than the expiration date of the patents-in-suit, including any extensions;

D. If DRL commercially manufactures, uses, offers to sell, or sells DRL's Generic Product within the United States, or imports DRL's Generic Product into the United States, prior to the expiration of the patents-in-suit including, any extensions, a Judgment awarding Plaintiffs monetary relief together with interest;

E. Attorneys' fees in this action as an exceptional case pursuant to 35 U.S.C. § 285;

F. Costs and expenses in this action; and

G. Such further and other relief as this Court may deem just and proper.



Dated: November 17, 2014

By: s/ Charles M. Lizza  
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*Attorneys for Plaintiff  
BioMarin Pharmaceutical Inc.*

**CERTIFICATION PURSUANT TO LOCAL CIVIL RULES 11.2 & 40.1**

Pursuant to Local Civil Rules 11.2 & 40.1, I hereby certify that the matter in controversy is not the subject of any other action pending in any court, or of any pending arbitration or administrative proceeding.

Dated: November 17, 2014

By: s/ Charles M. Lizza  
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*Attorneys for Plaintiff  
BioMarin Pharmaceutical Inc.*

# **EXHIBIT A**

US007566462B2

(12) **United States Patent**  
**Jungles et al.**(10) **Patent No.:** **US 7,566,462 B2**  
(45) **Date of Patent:** **Jul. 28, 2009**(54) **STABLE TABLET FORMULATION**(75) Inventors: **Steven Jungles**, Novato, CA (US);  
**Mark A. Henderson**, Larkspur, CA  
(US); **Victoria Sluzky**, Corte Madera,  
CA (US); **Robert Baffi**, Moraga, CA  
(US)(73) Assignee: **BioMarin Pharmaceutical Inc.**,  
Novato, CA (US)( \* ) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 0 days.(21) Appl. No.: **12/106,621**(22) Filed: **Apr. 21, 2008**(65) **Prior Publication Data**

US 2008/0207626 A1 Aug. 28, 2008

**Related U.S. Application Data**(63) Continuation of application No. 10/563,418, filed as  
application No. PCT/US2005/041252 on Nov. 16,  
2005.(60) Provisional application No. 60/629,189, filed on Nov.  
17, 2004.(51) **Int. Cl.**  
**A61K 9/20** (2006.01)(52) **U.S. Cl.** ..... **424/464**; 424/465(58) **Field of Classification Search** ..... 424/464,  
424/465

See application file for complete search history.

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*Primary Examiner*—Michael G. Hartley*Assistant Examiner*—James W. Rogers(74) *Attorney, Agent, or Firm*—Jones Day(57) **ABSTRACT**The present invention is directed to a stable solid formula-  
tions of tetrahydrobiopterin, processes for producing them,  
and treatment methods using such formulations.**20 Claims, 1 Drawing Sheet**

U.S. Patent

Jul. 28, 2009

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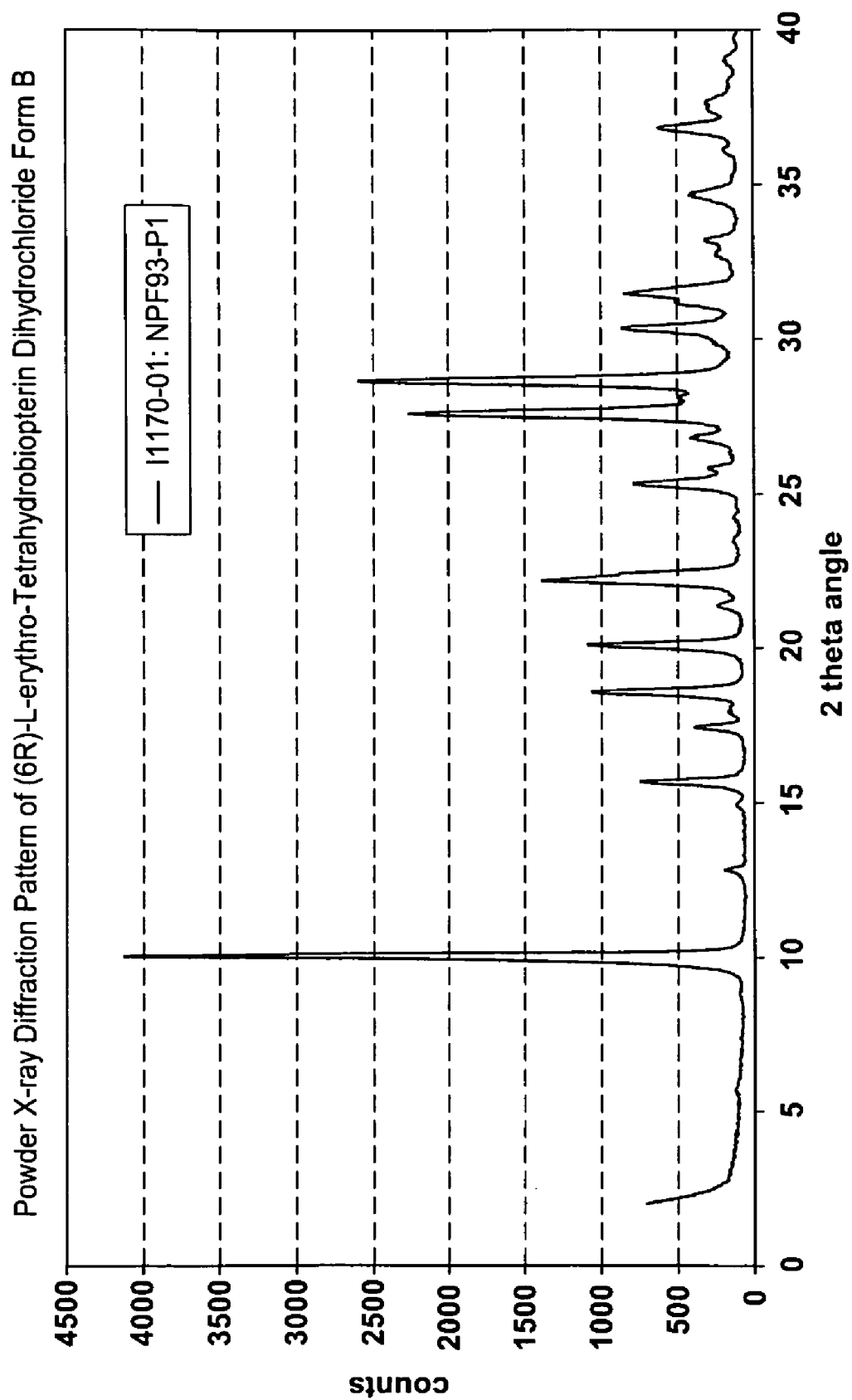


FIGURE 1

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**STABLE TABLET FORMULATION****CROSS REFERENCE TO RELATED APPLICATIONS**

This application is a continuation of U.S. Ser. No. 10/563, 418, which is a national stage application of International Application No. PCT/US05/41252, filed Nov. 16, 2005, which claims the benefit of U.S. Provisional Application No. 60/629,189, filed Nov. 17, 2004, the entirety of each is incorporated herein by reference.

**NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT**

BioMarin Pharmaceutical Inc. and Merck Eprova AG

**BACKGROUND****1. Field**

The present invention is generally directed to stable tablet formulations of tetrahydrobiopterin or precursors, derivatives or analogs thereof for the treatment of humans.

**2. Background of the Related Technology**

Tetrahydrobiopterin (sometimes referred to as BH4) is a biogenic amine of the naturally-occurring pterin family that is a cofactor for a number of different enzymes, including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. It is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (*Disorders of tetrahydrobiopterin and related biogenic amines*. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1275-1776).

A deficiency in PAH activity, due to absent or mutated PAH enzyme or a deficiency in its cofactor BH4, manifests as an excess of the amino acid phenylalanine (Phe) known as hyperphenylalaninemia (HPA) in its very mildest forms or phenylketonuria (PKU) in its moderate or severe forms. PAH deficiency also causes a deficiency in the amino acid tyrosine, which is a precursor for synthesis of neurotransmitters. A deficiency in tyrosine hydroxylase or tryptophan hydroxylase activity can manifest as a deficiency in neurotransmitter production.

Despite the elucidation of the role of BH4 deficiency in phenylketonuria, treatment with BH4 has not been suggested because such treatment is very expensive, as high as \$30,000 per year for an adolescent or adult, as compared with \$6,000 for phenylalanine-restricted dietary therapy (Hanley, N. *Engl. J. Med* 348(17):1723, 2003). Another significant problem with BH4 is that this compound is unstable and readily undergoes aerobic oxidation at room temperature (Davis et al., *Eur. J. Biochem.*, Vol 173, 345-351, 1988; U.S. Pat. No. 4,701, 455) and has a shelf-life of less 8 hours at room temperature (Bernegger and Blau, *Mol. Genet. Metabol.* 77:304-313, 2002).

Other tetrahydrobiopterin products available on the market need to be specially packaged or kept frozen. For example the labeling on the tablets sold by Schirck's Laboratory specify that the tablets should be kept frozen and state that the product has a shelf life at room temperature of only 2 months.

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BIOPTEN (tetrahydrobiopterin granules) requires expensive, hermetically-sealed foil packaging to maintain room temperature stability. The instability of such BH4 compositions is commercially undesirable and significant degradation due to improper storage could hinder therapy of patients.

Drug substance polymorphic forms can exhibit different physical and mechanical properties, including hygroscopicity, particle shape, density, flowability, and compactability, which in turn may affect processing of the drug substance and/or manufacturing of the drug product. The effect of polymorphism on pharmaceutical processing also depends on the formulation and the manufacturing process. Polymorphic forms of the drug substance can undergo phase conversion when exposed to a range of manufacturing processes, such as drying, milling, micronization, wet granulation, spray-drying, and compaction. Exposure to environmental conditions such as humidity and temperature can also induce polymorph conversion. The extent of conversion generally depends on the relative stability of the polymorphs, kinetic barriers for phase conversion, and applied stress. See FDA Center for Drug Evaluation and Research (CDER) Draft Guidance for Industry ANDAs: Pharmaceutical Solid Polymorphism Chemistry, Manufacturing, and Controls Information, December 2004.

Thus, there remains a need for a stable solid formulation of tetrahydrobiopterin and processes for manufacturing such stable formulations. The present invention is directed to addressing such a need.

**SUMMARY OF THE INVENTION**

The present invention relates to stable solid formulations of tetrahydrobiopterin, particularly stable tablets, processes for producing such formulations, and treatment methods using such formulations.

The invention provides a stable solid formulation of tetrahydrobiopterin, or a precursor or derivative or analog thereof, that maintains its stability for an extended period of time. Compositions of the invention may comprise a stable, crystalline form of BH4 that is stable at room temperature for more than 8 hours and a pharmaceutically acceptable carrier, diluent or excipient. Exemplary stable tablets of the invention have been prepared using a dry tableting process and have been shown to have a shelf-life of at least 6 to 9 months at room temperature.

Another aspect of the invention provides a dry formulation process for preparing stable solid formulations, which includes the step of mixing tetrahydrobiopterin, or a precursor or derivative or analog thereof, with another pharmaceutical carrier, diluent or excipient, in the absence of added water.

In an exemplary embodiment, the active pharmaceutical ingredient and excipients are dry blended and compressed. The tablets are processed in humidity-controlled rooms where humidity is kept at about 65% ( $\pm 5\%$ ) or less. Once processed, the tablets are stored in triple plastic lined water resistant containers with desiccant pillows lined between the outer two layers of plastic bags. Thus, the invention includes a dry formulation method comprising the steps of mixing an initial amount of a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin, preferably polymorph B, and one or more pharmaceutically acceptable excipients, and forming a tablet from the mixture, wherein the steps do not include adding liquid water. Exemplary particle sizes include, e.g., from about 0.2  $\mu\text{m}$  to about 500  $\mu\text{m}$ , from about 1  $\mu\text{m}$  to about 250  $\mu\text{m}$ , or from about 2  $\mu\text{m}$  to about 200  $\mu\text{m}$ , or, e.g., smaller

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than about 500  $\mu\text{m}$ , smaller than about 600  $\mu\text{m}$ , smaller than about 700  $\mu\text{m}$ , or smaller than about 850  $\mu\text{m}$ .

In exemplary embodiments, the tablet is initially manufactured using a stable crystalline form of (6R)-5,6,7,8-tetrahydrobiopterin described below as "polymorph B," and retains at least about 95% of the active pharmaceutical ingredient (API) at room temperature after 3 months, 6 months or 9 months, or preferably 12 months or longer, e.g. 15 months, 18 months, 21 months, 2 years, 2.5 years, 3 years or longer. Preferably the tablet retains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% of the API after storage at room temperature for such time periods. The tablet also preferably exhibits loss on drying of 2% or less, or 1.5% or less, or 1% or less, or 0.9% or less, or 0.8% or less, or 0.7% or less, or 0.6% or less, after such time periods. Exemplary tablets may be manufactured wherein the initial amount of tetrahydrobiopterin active pharmaceutical ingredient is about 25 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 550 mg, 600 mg, 650 mg, 700 mg, 750 mg, 800 mg, 850 mg, 900 mg or higher doses. Preferred tablets also exhibit rapid disintegration upon administration, e.g., 3 minutes or less, to improve ease of administration.

Thus, the invention provides a stable tablet formulation comprising an initial amount of a crystalline polymorph, designated polymorph B, of (6R)-L-erythro-tetrahydrobiopterin and a pharmaceutically acceptable excipient, wherein after six months at room temperature and about 60% humidity the stable tablet formulation retains at least about 95% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin, and wherein said crystalline polymorph, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 5.63 (m), 4.76 (m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w). Preferably the tablet retains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin.

The stable solid formulation preferably contains one or more of the following additional ingredients that improve stability or other characteristics of the formulation: binder, disintegrant, acidic antioxidant, or lubricant or combinations thereof. One exemplary preferred composition includes anhydrous dibasic calcium phosphate, crospovidone, ascorbic acid and stearyl fumarate, optionally with mannitol and riboflavin. The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or vitamins such as vitamin C and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan; and/or arginine. Compositions comprising tetrahydrobiopterin (or a precursor or derivative or analog) and a folate, and optionally further comprising arginine, are particularly contemplated.

The invention further contemplates other stable solid formulations for oral administration, e.g. capsules, pills or troches, with similar stability properties.

Yet another aspect of the invention provides treatment methods using such stable solid formulations. The invention contemplates that such formulations of the invention are useful for intervention in metabolic disorders, particularly those involving amino acid metabolism. More particularly, the stable formulations may be used for the treatment of subjects

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exhibiting elevated phenylalanine levels or decreased tyrosine levels, for example, subjects suffering from hyperphenylalanemia, mild phenylketonuria or classic severe phenylketonuria; and for the treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity, including vascular diseases, ischemic or inflammatory diseases, diabetes, or insulin resistance. The total dose required for each treatment may be administered in multiple doses or in a single dose. The stable formulations may be administered daily or at some other interval, e.g., every alternative day or even weekly.

The stable formulations may be used alone or in conjunction with other therapies suitable for the disorder to be treated, including the underlying disease or the clinical symptoms. For example, for HPA, the stable formulations of the invention may be administered in combination with a protein-restricted diet, e.g. where the subject is limited to about 600 mg or less, or about 300 mg or less of protein daily, optionally with supplemental amino acids, such as tyrosine, valine, isoleucine and leucine. The stable formulations may also be administered in combination with folates, arginine, vitamins, or neurotransmitter precursors. As another example, for vascular diseases, diabetes, or insulin resistance, the stable formulations of the invention may be administered in conjunction with other therapeutic agents such as anti-hypertensive agents, anti-platelet agents, cholesterol-lowering agents, insulin or oral hypoglycemic agents.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a characteristic X-ray powder diffraction pattern for crystalline polymorph B of (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a stable formulation that maintains a stable crystalline polymorph of the active ingredient. An anhydrous polymorph of (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride that is stable at room temperature to atmospheric oxygen and normal humidity, described below as polymorph B, has been identified. However, when the percent relative humidity approaches 80%, polymorph B appears to take up much more water, loses its crystalline form, and becomes labile to oxidation.

By using a dry formulation process, the stable crystalline structure of this polymorph is maintained in the finished product. In contrast, other processes for preparing tetrahydrobiopterin compositions result in a less stable product compared to those of the present invention.

Stable tablet formulations of the invention have been made using polymorph B in a dry formulation process and have been shown to retain 99% or more of the initial (6R)-5,6,7,8-tetrahydrobiopterin for at least 6 or 9 months, both at normal room temperature and humidity, and under accelerated testing conditions. The observed stability under accelerated testing conditions, i.e. higher temperature and humidity, indi-



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cates that the tablet formulations would be stable for far longer than 6 or 9 months at normal room temperature and humidity.

As used herein, "shelf life" means the storage period during which an active pharmaceutical ingredient (API) in a pharmaceutical formulation has minimal degradation (e.g., not more than about 5% degradation) when the pharmaceutical formulation is stored under specified storage conditions, for example, room temperature at normal humidity.

The shelf-life of the stable formulations of the invention may be measured as follows. The formulation to be tested may be divided into one or more different batches and stored under typical storage conditions, for example, 4° C. (refrigerator), or 25° C. (room temperature). Degradation of the API in a pharmaceutical formulation can also be detected using accelerated testing under exaggerated storage conditions designed to increase the degradation rate of the drug substance. For example, a batch can be "stressed" (placed in chamber which maintains a temperature of 45° C. and 75% humidity). Samples of each batch of formulation are then analyzed at different time points (e.g., time zero, 2 weeks, 1 month, 3 months, 6 months, 9 months, 1 year, 1.5 years, 2 years, 2.5 years, 3 years or longer) for amount of API still present in the formulation. Analysis of the API in the formulation may be carried out by a variety of detection methods including high performance liquid chromatography, crystal or powder X-ray diffraction, infrared or Raman spectra studies, microscopy, differential scanning calorimetry, thermal gravimetric analysis, hot-stage microscopy, and solid state nuclear magnetic resonance. Maintenance of a particular polymorph form can be determined by carrying out, e.g., powder or crystal X-ray diffraction studies or any of the same techniques used to analyze the polymorph initially.

#### I. SYNTHESIS OF TETRAHYDROBIOPTERIN, PRECURSORS, DERIVATIVES AND ANALOGS

A variety of methods are known in the art for synthesis of tetrahydrobiopterins, precursors, derivatives and analogs. U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408, Canadian application CA 2420374, European application nos. EP 079 574, EP 191 335 and Suntory Japanese patent publications JP 4-082888, JP 59-021685 and JP 9-157270, as well as Sugimoto and Matsuura, *Bull. Chem. Soc. Japan*, 48(12):3767-3768 (1975), Sugimoto and Matsuura, *Bull. Chem. Soc. Japan*, 52(1):181-183 (1979), Matsuura et al., *Chem. Lett. (Japan)*, 735-738 (1984), Matsuura et al., *Heterocycles*, Vol. 23, No. 12, 3115-3120, 1985 and Whiteley et al., *Anal Biochem.* 137(2):394-6 (1984) (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivatives thereof that may be used as compositions for the present invention.

Int'l Publication No. WO2005049614, U.S. Pat. No. 4,540,783, Japanese Patent No. 59-021685, Schircks et al., *Helv. Chim. Acta*, 60: 211 (1977), Sugimoto et al., *Bull. Chem. Soc. Jp*, 52(1):181 (1979), Sugimoto et al., *Bull. Chem. Soc. Jp*, 48(12):3767 (1975), Visontini et al., *Helv. Chim. Acta*, 52:1225 (1969), and Matsuura et al., *Chem. Lett.*, p 735 (1984), incorporated herein by reference in their entireties, describe methods of synthesizing BH4.

Nonlimiting examples of analogs for use in the compositions and methods described herein include pteridine, pterin, neopterin, biopterin, 7,8-Dihydrobiopterin, 6-methyltetrahydropterin, and other 6-substituted tetrahydropterin and other

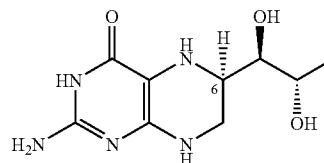
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6-substituted tetrahydropterins, sepiapterin, 6,7-Dimethyltetrahydropterin, 6-methyl biopterin and other 6-substituted biopterins, and other analogs that are described in the art. Nonlimiting examples of derivatives for use in the compositions and methods described herein include the derivatives described in U.S. Pat. Nos. 4,758,571; 4,774,244; 6,162,806; 5,902,810; 2,955,110; 2,541,717; 2,603,643; and 4,371,514, the disclosures of which are hereby incorporated herein.

Any such methods or other methods known in the art may be used to produce BH4, or precursors, derivatives or analogs for use in the stable formulations and therapeutic methods of the present invention.

#### II. CRYSTAL POLYMORPHS OF 6R-TETRAHYDROBIOPTERIN HYDROCHLORIDE SALT

It has been found that BH4, and in particular, the dihydrochloride salt of BH4, exhibits crystal polymorphism. The structure of BH4 is shown below:



The (6R) form of BH4 is the known biologically active form, however, BH4 is known to be unstable at ambient temperatures.

BH4 is difficult to handle and it was therefore produced and offered as its dihydrochloride salt (Schircks Laboratories, Jona, Switzerland) in ampoules sealed under nitrogen to prevent degradation of the substance due to its hygroscopic nature and sensitivity to oxidation. U.S. Pat. No. 4,649,197 discloses that separation of (6R)- and (6S)-L-erythro-tetrahydrobiopterin dihydrochloride into its diastereomers is difficult due to the poor crystallinity of (6R,S)-L-erythro-tetrahydrobiopterin dihydrochloride. The European patent number 0 079 574 describes the preparation of tetrahydrobiopterin, wherein a solid tetrahydrobiopterin dihydrochloride is obtained as an intermediate. S. Matsuura et al. describes in *Chemistry Letters* 1984, pages 735-738 and *Heterocycles*, Vol. 23, No. 12, 1985 pages 3115-3120 (6R)-tetrahydrobiopterin dihydrochloride as a crystalline solid in form of colorless needles, which are characterized by X-ray analysis disclosed in *J. Biochem.* 98, 1341-1348 (1985). An optical rotation of 6.81° was found the crystalline product, which is quite similar to the optical rotation of 6.51° reported for a crystalline solid in form of white crystals in example 6 of EP-A2-0 191 335.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride indicated that the compound may exist in different crystalline forms, including polymorphic forms and solvates. It has been found that one crystal polymorph of BH4 is more stable, and is stable to decomposition under ambient conditions.

#### Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B."

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Further-



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more, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties renders polymorph form B especially feasible for pharmaceutical applications, which are often prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride. As used herein, the following the abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity.

Form B can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive crystal form B, can be used for the preparation of the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms, in suitable polar and non aqueous solvents.

Other forms of BH4 can be converted into form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methyl-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallisation from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water:acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a

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similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallization from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallisation and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application.

### III. STABLE PHARMACEUTICAL FORMULATIONS

Pharmaceutical formulations may initially include a stable crystalline form of tetrahydrobiopterin, or a precursor or derivative or analog thereof, with a pharmaceutically acceptable carrier. The stable formulation of the invention preferably contains one or more of the following additional ingredients that improve the stability or other characteristics of the formulation: binder, disintegration agent, acidic antioxidant, or lubricant or combinations thereof. Preferably a stable tablet formulation includes a binder and disintegration agent, optionally with an acidic antioxidant, and optionally further including a lubricant.

The initial amount of a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin used to prepare the formulation may be, for example, in the range of about 30 wt % to about 40 wt % of the formulation, or in the range of about 32 wt % to about 35 wt %, or at about 33 wt %.

Binders assist in maintaining the tablet formulation. In some cases, anhydrous binders are used to preserve the anhydrous state of polymorph B. In some cases, the binder may act as a drying agent. Exemplary binders include anhydrous dibasic calcium phosphate and its monohydrate.

Exemplary concentrations of the binder in a stable tablet formulation of the present invention are between about 1 wt % to about 5 wt %. Particularly contemplated concentrations are between about 1.5 and 3 wt %. Also contemplated are con-

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centrations of binder of at least about 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %, or concentrations up to about 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, and 5.0 wt %. The weight ratio of binder to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:10 to about 1:20. Also contemplated are weight ratios of about 1:10.25, 1:10.5, 1:10.75, 1:11, 1:11.25, 1:11.5, 1:11.75, 1:12, 1:12.25, 1:12.5, 1:12.75, 1:13, 1:13.25, 1:13.5, 1:13.75, 1:14, 1:14.25, 1:14.5, 1:14.75, 1:15, 1:15.25, 1:15.5, 1:15.75, 1:16, 1:16.25, 1:16.5, 1:16.75, 1:17, 1:17.25, 1:17.5, 1:17.75, 1:18, 1:18.25, 1:18.5, 1:18.75, 1:19, 1:19.25, 1:19.5, and 1:19.75.

Disintegration agents assist in rapid disintegration of solid formulations by absorbing water and expanding. Exemplary disintegration agents include polyvinylpyrrolidone (PVP, e.g. sold under the name POVIDONE), a cross-linked form of povidone (CPVP, e.g. sold under the name CROSPOLV-DONE), a cross-linked form of sodium carboxymethylcellulose (NaCMC, e.g. sold under the name AC-DI-SOL), other modified celluloses, and modified starch. Tablets formulated with CPVP exhibited much more rapid disintegration than tablets formulated with PVP.

Exemplary concentrations of the disintegration agent in a stable tablet formulation of the present invention are between about 1 wt % to about 20 wt %. Particularly contemplated concentrations are between about 3 wt % and about 10 wt %. Also contemplated are concentrations of disintegration of at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %, or concentrations up to about 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.2, 5.4, 5.6, 5.7, 5.8, 6.0, 6.25, 6.5, 6.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5, 9.75, and 10.0 wt %. The weight ratio of disintegration agent to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:5 to about 1:10. Also contemplated are weight ratios of about 1:5.25, 1:5.5, 1:5.75, 1:6.0, 1:6.25, 1:6.5, 1:6.75, 1:7.0, 1:7.25, 1:7.5, 1:7.75, 1:8.0, 1:8.25, 1:8.5, 1:8.75, 1:9.0, 1:9.25, 1:9.5, and 1:9.75.

Antioxidants may be included and help stabilize the tetrahydrobiopterin product, especially after dissolution. Low pH aqueous solutions of API are more stable than are solutions at high pH. Exemplary acidic antioxidants include ascorbic acid, fatty acid esters of ascorbic acid such as ascorbyl palmitate and ascorbyl stearate, and salts of ascorbic acid such as sodium, calcium, or potassium ascorbate. Non-acidic antioxidants may also be used in the stable tablet formulations. Nonlimiting examples of non-acidic antioxidants include beta-carotene, alpha-tocopherol. Acidic additives may be added to enhance stability of the tablet formulation, including citric acid or malic acid.

Exemplary concentrations of the antioxidant in a stable tablet formulation of the present invention are between about 1 wt % and about 3 wt %. Particularly contemplated concentrations are at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, and 2.0 wt %, or concentrations up to about 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %. The weight ratio of antioxidant to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:5 to 1:30. Also contemplated are weight ratios of about 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5, 1:10, 1:10.5, 1:11, 1:11.5, 1:12, 1:12.5, 1:13, 1:13.5, 1:14, 1:14.5, 1:15, 1:15.5, 1:16, 1:16.5, 1:17, 1:17.5, 1:18, 1:18.5, 1:19, 1:19.5, 1:20, 1:20.5, 1:21, 1:21.5, 1:22, 1:22.5, 1:23, 1:23.5, 1:24, 1:24.5, 1:25, 1:25.5, 1:26, 1:26.5, 1:27, 1:27.5, 1:28, 1:28.5, 1:29, and 1:29.5.

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In Schirk's Laboratory tablets, ascorbic acid is present at a ratio of 1:1 to BH4. In contrast, the concentration of ascorbic acid in the stable formulations of the invention is far lower, e.g. a weight ratio (mg/mg) of 1:20 of ascorbic acid to BH4. Thus, the invention also contemplates formulations comprising ascorbic acid at a ratio to BH4 of less than 1:1, e.g. 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18 or 1:19, preferably a ratio of less than 1:10.

Lubricants improve stability, hardness and uniformity of solid formulations. Exemplary lubricants include stearyl fumarate and magnesium stearate.

Exemplary concentrations of the lubricant in a stable tablet formulation of the present invention are between about 0.1 wt % and about 2 wt %. Particularly contemplated concentrations are between about 0.5 and 1 wt %. Also contemplated are concentrations of lubricant of at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 wt %, or concentrations up to about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, and 2.0 wt %. The weight ratio of lubricant to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:25 to 1:65. Also contemplated are weight ratios of about 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:41, 1:42, 1:43, 1:44, 1:45, 1:46, 1:47, 1:48, 1:49, 1:50, 1:51, 1:52, 1:53, 1:54, 1:55, 1:56, 1:57, 1:58, 1:59, 1:60, 1:61, 1:62, 1:63, 1:64, and 1:65.

The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or arginine; and/or vitamins, such as vitamin C and/or vitamin B 2 (riboflavin) and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan.

Exemplary folates, including folate precursors, folic acids, or folate derivatives, are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and pharmaceutically acceptable salts thereof. Exemplary salts include sodium, potassium, calcium or ammonium salts.

Exemplary relative weight ratios of BH4 to folates to arginine may be from about 1:10:10 to about 10:1:1.

Optionally the stable formulations of the invention can also comprise other excipients such as mannitol, hydroxypropyl cellulose, microcrystalline cellulose, or other non-reducing sugars such as sucrose, trehalose, melezitose, planteose, and raffinose. Reducing sugars may react with BH4.

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Pharmaceutically acceptable ingredients for manufacturing solid formulations for oral administration may be incorporated, for example, by admixing the components and optionally finely dividing them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches. Coatings may be applied after compression to form pills.

Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be for example binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. Nonlimiting examples of binders useful in a composition described herein include gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (polyoxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Nonlimiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate. Nonlimiting examples of lubricants use in a composition described herein include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Surfactants for use in a composition described herein can be anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described

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herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The polymorph described herein may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. Slow release formulations may also be prepared from the polymorph described herein in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used. Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4 aminosalicylic acid, 2 phenoxybenzoic acid, 2 acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzene-sulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2 sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.



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The stable formulations of the invention may be provided, e.g. as tablets or pills or capsules in HDPE bottles provided with a desiccant capsule or pouch; or in foil-on-foil blister packaging, or in blister packaging comprising see-through polymer film, if commercially desirable.

The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey ducks and geese.

#### IV. TREATMENT METHODS USING STABLE FORMULATIONS

The stable formulations of the invention may be used for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine or tryptophan levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal. Conditions associated with decreased tyrosine or tryptophan levels include neurotransmitter deficiency, neurological and psychiatric disorders such as Parkinson's, dystonia, spinocerebellar degeneration, pain, fatigue, depression, other affective disorders and schizophrenia.

The stable formulations may also be used for treating patients suffering from BH4 deficiency, e.g., due to a defect in the pathway for its synthesis, including but not limited to dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, or dihydropteridine reductase (DHPR) deficiency.

Suitable subjects for treatment with the stable formulations of the invention include subjects with an elevated plasma Phe concentration in the absence of the therapeutic, e.g. greater than 1800  $\mu\text{M/L}$ , or greater than 1600  $\mu\text{M}$ , greater than 1400  $\mu\text{M}$ , greater than 1200  $\mu\text{M}$ , greater than 1000  $\mu\text{M}$ ,

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greater than 800  $\mu\text{M}$ , or greater than 600  $\mu\text{M}$ , greater than 420  $\mu\text{M}$ , greater than 300  $\mu\text{M}$ , greater than 200  $\mu\text{M}$ , or greater than 180  $\mu\text{M}$ . Mild PKU is generally classified as plasma Phe concentrations of up to 600  $\mu\text{M/L}$ , moderate PKU as plasma Phe concentrations of between 600  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$  and classic or severe PKU as plasma Phe concentrations that are greater than 1200  $\mu\text{M/L}$ . Preferably treatment with the stable formulations alone or with protein-restricted diet decreases the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ , or less than 500  $\mu\text{M}$ , or 360  $\mu\text{M} \pm 15 \mu\text{M}$  or less, or less than 200  $\mu\text{M}$ , or less than 100  $\mu\text{M}$ . Other suitable subjects include subjects diagnosed as having a reduced phenylalanine hydroxylase (PAH) activity. Reduced PAH activity may result from a mutation in the PAH enzyme, for example, a mutation in the catalytic domain of PAH or one or more mutations selected from the group consisting of F39L, L48S, 165T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241c, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C; or subjects that are pregnant females, females of child-bearing age that are contemplating pregnancy, or infants between 0 and 3 years of age, or 0-2, 0-1.5 or 0-1; or subjects diagnosed as unresponsive within 24 hours to a single-dose BH4 loading test or a multiple dose loading test, such as a 4-dose or 7-day loading test. Exemplary patient populations and exemplary BH4 loading tests are described in Int'l. Publication No. WO 2005/049000, incorporated herein by reference in its entirety.

U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment as described herein.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg  $\times$  5 kg body weight) to 3000 mg/day (30 mg/kg  $\times$  100 kg body weight). While continuous, daily administration is contemplated, for HPA it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again. Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data.

In preferred embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

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The invention further contemplates that stable formulations of the invention may be used for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from co-treatment with folates, including folate precursors, folic acids, or folate derivatives.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (narrowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, such as hypertension, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, peripheral circulation disorder, coronary arteriovenous anastomosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arteriosclerotic heart diseases or the like so far as they are associated with insulin resistance. It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves

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adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

## V. COMBINATION THERAPY

Certain methods of the invention involve the combined use of the stable formulations of the invention and one or more other therapeutic agents.

In such combination therapy, administration of the stable formulations of the invention may be concurrent with or may precede or follow the administration of the second therapeutic agent, e.g. by intervals ranging from minutes to hours, so long as both agents are able to exert their therapeutic effect at overlapping time periods. Thus, the invention contemplates the stable formulations of the invention for use with a second therapeutic agent. The invention also contemplates use of a second therapeutic agent in preparation of a medicament for administration with the stable tetrahydrobiopterin, precursor, derivative or analog formulations of the invention.

Tetrahydrobiopterin therapy may be combined with dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. For example, one could administer to the subject the BH4 composition and a low-phenylalanine medical protein composition in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the

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BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, with a delay time of only about 1 hour being most preferred. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Gent. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4)243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

## VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Preparation of Stabilized Crystallized Form of BH4

Int'l. Publication No. WO 2005/065018, incorporated herein by reference in its entirety, describes X ray and Raman spectra studies to characterize the polymorphs, including hydrates or solvates, of BH4, as well as exemplary crystallization conditions under which the polymorphs can be prepared. Int'l. Publication No. WO 2005/049000, incorporated

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herein by reference in its entirety, describes various patient populations for which BH4 treatment is suitable and describes methods for treating such subjects with BH4. Int'l. Publication No. WO2005/049614 incorporated herein by reference in its entirety, describe methods of synthesizing BH4.

The references cited herein throughout, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.

### Example 2

#### Stable Tablet Formulation of Tetrahydrobiopterin

Three tablet formulations were prepared by mixing the ingredients shown in Table I as described in detail below.

TABLE I

Ingredient	Formulation I (wt %)	Formulation II (wt %)	Formulation III (wt %)
6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt, polymorph B (Active Ingredient)	33.33	33.33	33.33
D-Mannitol (Taste Masking)	57.56	57.56	57.56
Dibasic Calcium Phosphate, Anhydrous (Binder)	2.18	2.18	2.18
Hydroxypropyl Cellulose (Disintegrant)	3.63	4.5	
Polyvinylpyrrolidone (Disintegrant)	0.87		4.50
Ascorbic acid (Stabilizer)	1.67	1.67	1.67
Riboflavin (Coloring Agent)	0.01	0.01	0.01
Sodium Stearyl Fumarate (Lubricant)	0.75	0.75	0.75

For each formulation in Table I, twelve kilogram batches were prepared by first charging 4 kg of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt (SAPROPTERIN Hydrochloride, available from Daiichi Suntory Pharma Co., Ltd., Japan) to a blender and blending the BH4 for 10 minutes at 25 revolutions per minute (RPM). Then 6.91 kg of D-Mannitol (PEARLITOL, available from Roquette America, Inc., Keokuk, Iowa) was added to the blender and the mixture was allowed to blend for an additional 10 minutes at 25 RPM. Then 260 grams of Anhydrous Dibasic Calcium Phosphate (available from Mallinckrodt Baker, Inc., Phillipsburg, N.J.) and (a) in Formulation I, 436 grams of Hydroxypropyl Cellulose and 104 grams were added to the blender, (b) in Formulation II 540 grams of Hydroxypropyl Cellulose was added to the blender; (c) in Formulation III, 540 grams of Polyvinylpyrrolidone (KOLLIDON CL, available from BASF Corporation, Florham Park, N.J.) were added to the blender, and the mixture was allowed to blend for an additional 10 minutes at 25 RPM. To the blender 200 grams of Ascorbic Acid and 120 grams of Riboflavin were added to the blender and the mixture was allowed to blend for 3 minutes at 25 RPM. The Sodium Stearyl Fumarate lubricant (PRUV, available from Penwest Pharmaceuticals Co., Danbury, Conn.) was filtered through a 25 mesh stainless steel screen and into a bag, and the blender was then charged with 9 kg of the screened Sodium Stearyl Fumarate, and the resulting mixture was allowed to blend for 5 minutes at 25 RPM.

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The blended mixture of each formulation were then removed from the blender, and three samples of each formulation were collected for the preparation of a 150 mg, a 300 mg, and a 600 mg tablets. For each formulation, the tablet samples (150 mg, 300 mg, and 600 mg) were placed in a tablet press (available from Jenn-Chiang Mahinery Co., Ltd., Taiwan, R.O.C.) wherein the parameters of the tablet press were set to provide tablets with a thickness in the range of 4.5 to 5.5 millimeters, and a target hardness of 7 KP.

The resulting tablets were then analyzed to determine the stability of the formulations. The stability of the formulations was studied for a change in appearance over time by a visual inspection at different intervals, for disintegration of the formulation utilizing the United States Pharmacopeia recommendations no. 701, and for a chemical change by assaying the components of the formulations. The results of the stability tests are summarized below in Table II.

TABLE II

Form.	Test	Initial	2 weeks	4 weeks	8 weeks
I	Appearance	Off white, round Tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablet
	Disintegration	5 min 20 sec	5 min 40 sec	8 min 4 sec	—
	Chemical Assay	97.10%	97.90%	98.2	100.7
II	Appearance	Off white round Tablets	Broken chip on some tablets. Light yellow spots (diameter 1-2 mm) on the off white round tablets.	Broken chip on some tablets. Light yellow spots (diameter 1-2 mm) on the off white round tablets	Broken chip on some tablets. Light brown spots (diameter 1-2 mm) on the dark brown tablets
	Disintegration	4 min 10 sec	4 min 38 sec	6 min 52 sec	—
	Chemical Assay	102.70%	100.00%	100.2	97.4
III	Appearance	Color is off white	Rough surface, and color is light yellow	Rough surface, and color is light yellow	Rough surface, and color is yellow
	Disintegration	1 min 52 sec	35 sec	58 sec	—
	Chemical Assay	100.20%	102.90%	97.4	99.8

The stability tests show that tablet Formulation III is more stable than the other formulations of BH4. Each of the pharmaceutical preparations are useful formulations for the delivery of BH4. Formulation III exhibited better stability than Formulations I and II. Thus, in one preferred embodiment, the stabilized tablet formulation comprises an optimal disintegration agent, for example, crossprovidone or a disintegration agent more similar to polyvinylpyrrolidone than hydroxypropylcellulose. The preferred formulation is Formulation III. Other suitable tablet formulations may include at least ascorbic acid at a concentration of at least 0.01% weight, or at least 0.05% weight or at least 0.1% weight.

## Example 3

Three hundred mg tablets containing 100 mg tetrahydrobiopterin are prepared using the desired initial amount of polymorph B and mixing with other ingredients in the relative

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amounts shown below in Table III using the following dry tableting process. Tablets containing other desired amounts of tetrahydrobiopterin can be prepared in a similar manner.

The 6R-erythro-5,6,7,8-tetrahydrobiopterin and D-Mannitol were hand screened with a 20 mesh screen (designed to filter out particles greater than ~850  $\mu$ m in size) and placed in a blender. The mixture was blended for 10 minutes at 21 RPM. Next, the Anhydrous Dibasic Calcium Phosphate and CROSPVIDONE were hand screened with a 20 mesh screen and blended with the BH4 and D-Mannitol for 10 minutes at 21 RPM. The Ascorbic Acid and Riboflavin were added to the blender after hand screening with a 20 mesh screen, and the resulting mixture blended for 10 minutes at 21 RPM. Next, the Sodium Stearyl Fumarate was added to the blender after hand screening with a 40 mesh screen, and

blended for 5 minutes at 21 RPM. The blended mixture was then discharged into a bag and tested for uniformity, before being pressed into tablets.

TABLE III

Ingredient	(wt %)	(mg)
6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt (Active Ingredient)	33.33	100.0
D-Mannitol (Taste Masking)	57.56	172.68
Dibasic Calcium Phosphate, Anhydrous (Binder)	2.18	6.54
CROSPVIDONE (Disintegration Agent)	4.50	13.50
Ascorbic acid (Acidic Antioxidant)	1.67	5.00
Riboflavin	0.01	0.03



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TABLE III-continued

Ingredient	(wt %)	(mg)
(Coloring Agent)		
Stearyl Fumarate	0.75	2.25
(Lubricant)		

The tablets were packaged either in foil blister-packs, or in HDPE bottles in quantities of 45-tablets per bottle. Each type of packaged tablets were divided into two batches. One batch was stored at room temperature,  $25 \pm 2^\circ \text{C}$ ., and  $60 \pm 5\%$  rela-

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tive humidity. The other batch was stored under accelerated testing conditions, at  $40 \pm 2^\circ \text{C}$ . and  $75 \pm 5\%$  relative humidity. At regular intervals, tablets were removed from storage and tested for retention of the active pharmaceutical ingredient, 6R-L-erythro-5,6,7,8-tetrahydrobiopterin. Exemplary results for the 300 mg tablets are shown below in Tables IV, V, VI and VII. After six months storage under room temperature or accelerated testing conditions, each of the four batches showed retention of at least 99% of the original amount of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin by HPLC assay, loss on drying of less than 1%, and rapid disintegration in 3 minutes or less.

TABLE IV

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, FOIL BLISTER-PACK) STORAGE CONDITIONS: $25 \pm 2^\circ \text{C}$ ./ $60 \pm 5\%$ RH						
Test/Assay	Stability Specification	Time Point (months)				
		0	1	3	6	9
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	Conforms	Conforms
Loss on Drying	Report Result	0.3%	0.3%	0.9%	0.7%	0.9%
Disintegration	$\leq 3$ minutes	1 min. 30 sec.	2 min. 7 sec.	2 min. 2 sec.	2 min. 35 sec.	1 min. 48 sec.
HPLC Assay	90-110%	101%	100%	100%	100%	101%
HPLC for Related Substances	$\leq 0.1\%$ individual unidentified	0.05%	0.04% (RRT 1.28)	0.05% (RRT 0.72) (RRT 0.83) (RRT 1.32)	0.09% (RRT 0.81) (RRT 0.83) (RRT 1.24)	0.03% (RRT 0.69) (RRT 0.73) (RRT 0.81) 0.03% (RRT 0.83) 0.02% (RRT 1.21) 0.04% (RRT 1.44)
	$\leq 0.5\%$ total unidentified	0.05%	0.04%	0.18%	0.22%	0.20%
	$\leq 2.0\%$ total related substances	0.05%	0.04%	0.18%	0.22%	0.20%

ND = None detected

TABLE V

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, FOIL BLISTER-PACK) STORAGE CONDITIONS: $40 \pm 2^\circ \text{C}$ ./ $75 \pm 5\%$ RH					
Test/Assay	Stability Specification	Time Point (months)			
		0	1	3	6
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	Conforms
Loss on Drying	Report Result	0.3%	0.3%	0.8%	0.7%
Disintegration	$\leq 3$ minutes	1 min. 30 sec.	2 min. 16 sec.	2 min. 47 sec.	2 min. 11 sec.
HPLC Assay	90-110%	101%	100%	101%	99%
HPLC for Related Substances	$\leq 0.1\%$ individual unidentified	0.05%	0.04% (RRT 1.28)	0.06% (RRT 0.64) (RRT 0.72) 0.04% (RRT 0.83)	0.07% (RRT 0.74) (RRT 0.83) 0.04% (RRT 0.83) 0.06% (RRT 1.31)
					0.15% (RRT 1.24)



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TABLE V-continued

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, FOIL BLISTER-PACK) STORAGE CONDITIONS: 40 ± 2° C./75 ± 5% RH					
Test/Assay	Stability Specification	Time Point (months)			
		0	1	3	6
	≤0.5% total unidentified	0.05%	0.04%	0.23%	0.32%
	≤2.0% total related substances	0.05%	0.04%	0.23%	0.32%

ND = None detected

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TABLE VI

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45 TABLET BOTTLES) STORAGE CONDITIONS: 25 ± 2° C./60 ± 5% RH					
Test/Assay	Stability Specification	Time point (months)			
		0	3	6	
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	
Loss on Drying	Report Result	0.7%	0.8%	1.1%	
Disintegration	≤3 minutes	2 min 57 sec	2 min 3 sec	2 min 50 sec	
HPLC Assay	90-110%	102%	NT	103%	
HPLC for Related Substances	≤0.1% individual unidentified	0.06%	NT	0.08% (RRT 0.56) 0.04% (RRT 0.61) 0.05% (RRT 0.65) 0.04% (RRT 0.81)	
	≤0.5% total unidentified	0.15%	NT	0.21%	
	≤2.0% total related substances	0.15%	NT	0.21%	
	Report Result (mg/tablet)	5	NT	5	
	Total Aerobic Microbial Count	<100 cfu/g	NR	NR	
	≤ 1000 cfu/g				
	Test for <i>Escherichia coli</i> = absent	Absent			

ND = None detected

NT = Not tested

NR = Not required

TABLE VII

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45-TABLET BOTTLES) STORAGE CONDITIONS: 40 ± 2° C./75 ± 5% RH					
Test/Assay	Stability Specification	Time Point (months)			
		0	3	6	
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	
Loss on Drying	Report Result	0.7%	1.1%	1.1%	
Disintegration	≤3 minutes	2 min 57 sec	2 min 41 sec	2 min 48 sec	
HPLC Assay	90-110%	102%	NT	101%	

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TABLE VII-continued

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45-TABLET BOTTLES) STORAGE CONDITIONS: 40 ± 2° C./75 ± 5% RH					
Test/Assay	Stability Specification	Time Point (months)			
		0	3	6	
HPLC for Related Substances	≤0.1% individual unidentified	0.06%	NT	0.13% (RRT 0.56) 0.03% (RRT 0.61) 0.10% (RRT 0.65) 0.04% (RRT 0.81)	
	≤0.5% total unidentified	0.15%	NT	0.30%	
	≤2.0% total related substances	0.15%	NT	0.30%	
	Report Result (mg/tablet)	5	NT	5	

ND = None detected

NT = Not tested

NR = Not required

What is claimed is:

1. A stable tablet formulation comprising an initial amount of a crystalline polymorph, designated polymorph B, of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, an antioxidant, and a pharmaceutically acceptable excipient, diluent, or carrier in the form of a tablet,

wherein the weight ratio of the antioxidant to the (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is about 1:5 to about 1:30;

wherein after six months in a container at room temperature and about 60% humidity the stable tablet formulation retains at least about 95% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, and wherein said crystalline polymorph B exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 5.63 (m), 4.76 (m), 4.40 (m), 4.00 (s), 3.23 (s), and 3.11 (vs).

2. The stable tablet formulation of claim 1, wherein the crystalline polymorph B further exhibits characteristic peaks expressed in d-values (Å): 6.9 (w), 5.07 (m), 4.15 (w), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.17 (w), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w).

3. The stable tablet formulation of claim 1, wherein after six months in a container at room temperature and about 60%

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humidity the stable tablet formulation retains at least about 98% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin.

4. The stable tablet formulation of claim 1, wherein after nine months in a container at room temperature and about 60% humidity the stable tablet formulation retains at least about 95% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin.

5. The stable tablet formulation of claim 1, wherein after twelve months in a container at room temperature and about 60% humidity the stable tablet formulation retains at least about 95% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin.

6. The stable tablet formulation of claim 1, wherein the initial amount of (6R)-L-erythro-tetrahydrobiopterin is in a range of about 30 wt % to about 40 wt % of the formulation.

7. The stable tablet formulation of claim 1, wherein the initial amount of (6R)-L-erythro-tetrahydrobiopterin in a tablet is selected from the group consisting of 100 mg, 200 mg, and 300 mg.

8. The stable tablet formulation of claim 1, wherein the initial amount of (6R)-L-erythro-tetrahydrobiopterin in a tablet is 400 mg.

9. The stable tablet formulation of claim 1, further comprising a disintegration agent.

10. The stable tablet formulation of claim 9, wherein the disintegration agent is croscopovidone.

11. The stable tablet formulation of claim 1, further comprising a lubricant.

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12. The stable tablet formulation of claim 11, wherein the lubricant is stearyl fumarate.

13. The stable tablet formulation of claim 12, wherein the stearyl fumarate is sodium stearyl fumarate.

14. The stable tablet formulation of claim 11, wherein the lubricant is present in a range of about 1.0 wt % to about 1.8 wt % of the formulation.

15. The stable tablet formulation of claim 12, further comprising microcrystalline cellulose.

16. The stable tablet formulation of claim 1, comprising an initial amount of the (6R)-L-erythro-tetrahydrobiopterin of about 400 mg, croscopovidone in a range of about 3 wt % to about 5 wt % of the formulation, and stearyl fumarate in a range of about 1 wt % to about 1.7 wt % of the formulation.

17. The stable tablet formulation of claim 1, comprising an initial amount of the (6R)-L-erythro-tetrahydrobiopterin in a range of about 32 wt % to about 35 wt % of the formulation, croscopovidone in a range of about 3 wt % to about 5 wt % of the formulation, anhydrous dibasic calcium phosphate in a range of about 1.5 wt % to about 3 wt % of the formulation, and stearyl fumarate in a range of about 0.5 wt % to about 2 wt % of the formulation.

18. The stable tablet formulation of claim 1, wherein the antioxidant is an acidic antioxidant.

19. The stable tablet formulation of claim 1, wherein the antioxidant is ascorbic acid.

20. The stable tablet formulation of claim 19, wherein the weight ratio of ascorbic acid to the (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is about 1:20.

\* \* \* \* \*

# **EXHIBIT B**

US007566714B2

(12) **United States Patent**  
**Oppenheimer et al.**

(10) **Patent No.:** **US 7,566,714 B2**

(45) **Date of Patent:** **Jul. 28, 2009**

(54) **METHODS AND COMPOSITIONS FOR THE TREATMENT OF METABOLIC DISORDERS**

(56) **References Cited**

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(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(22) Filed: **Jun. 1, 2005**

(Continued)

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(63) Continuation of application No. 10/991,573, filed on Nov. 17, 2004, now abandoned.

(60) Provisional application No. 60/520,767, filed on Nov. 17, 2003.

(57) **ABSTRACT**

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**A61K 31/495** (2006.01)

(52) **U.S. Cl.** ..... **514/249**; 514/250

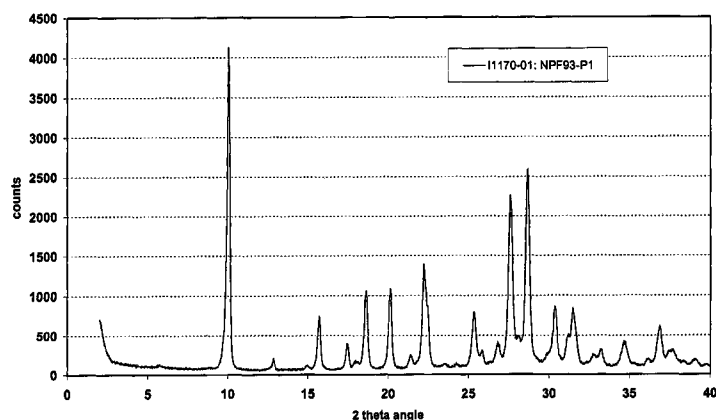
(58) **Field of Classification Search** ..... 514/183,  
514/249, 250

The present invention is directed to a novel methods and compositions for the therapeutic intervention in hyperphenylalaninemia. More specifically, the specification describes methods and compositions for treating various types of phenylketonurias using compositions comprising BH4. Combination therapies of BH4 and other therapeutic regimens are contemplated.

See application file for complete search history.

**46 Claims, 20 Drawing Sheets**

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form B



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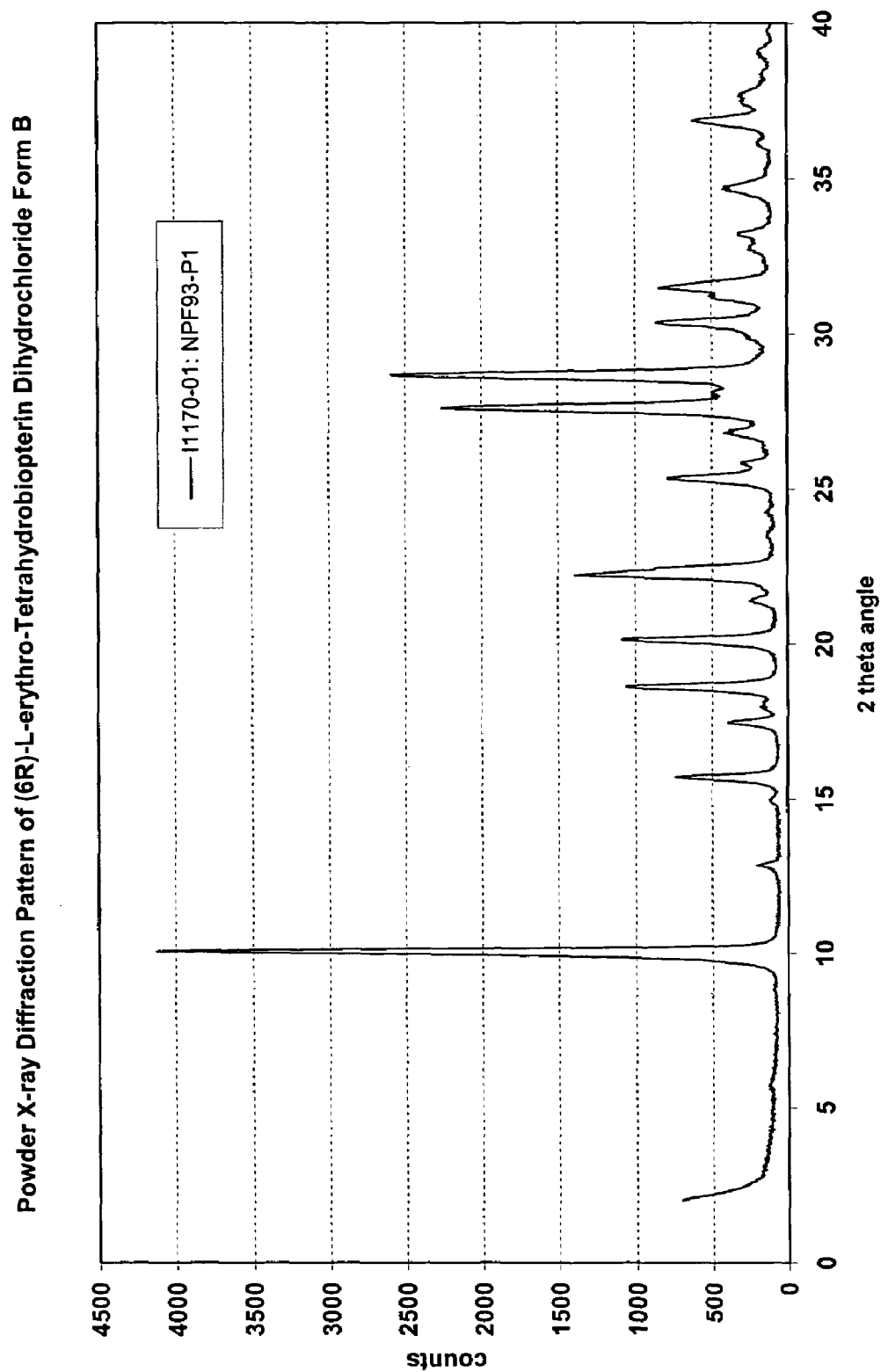


Figure 1

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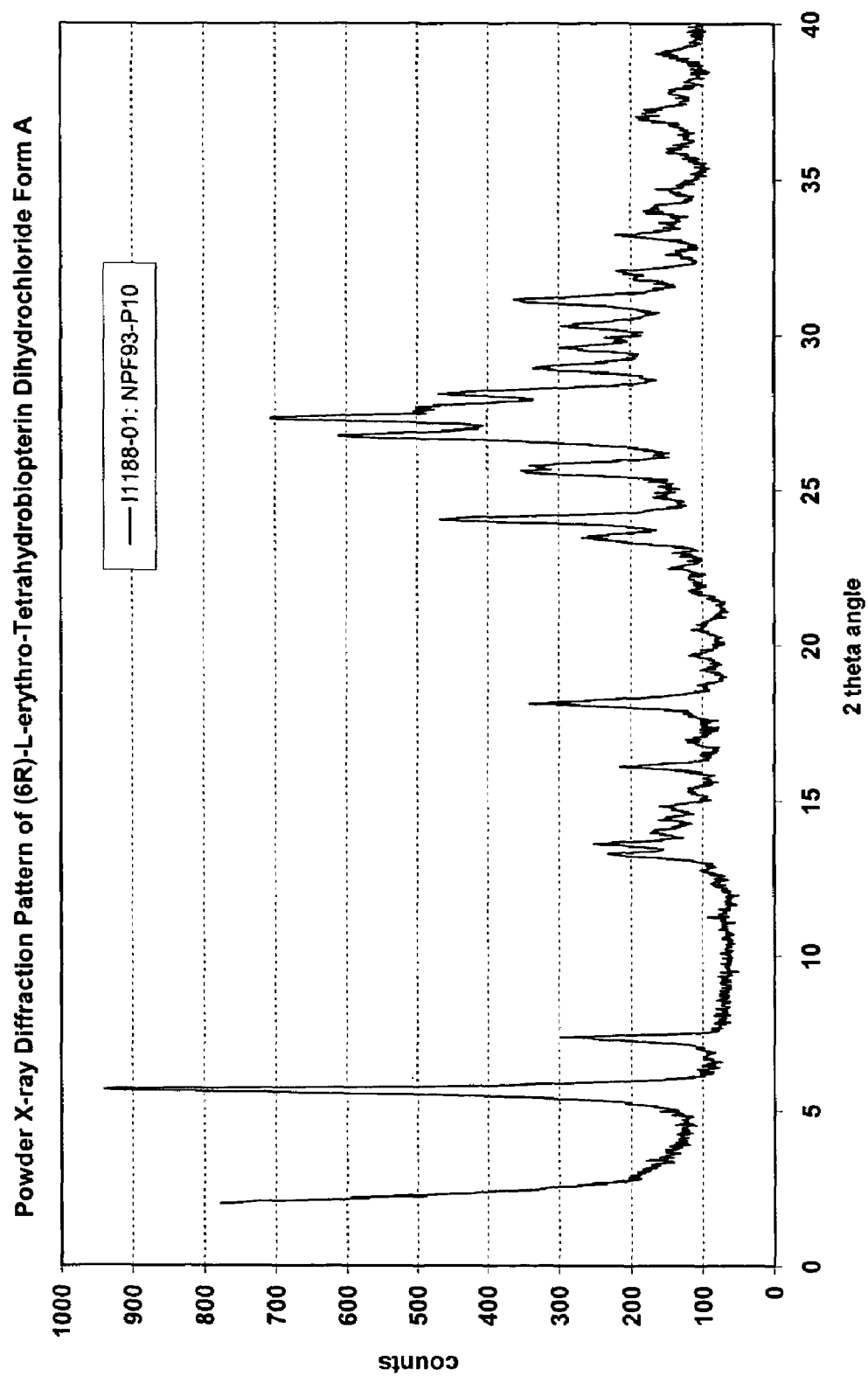


Figure 2

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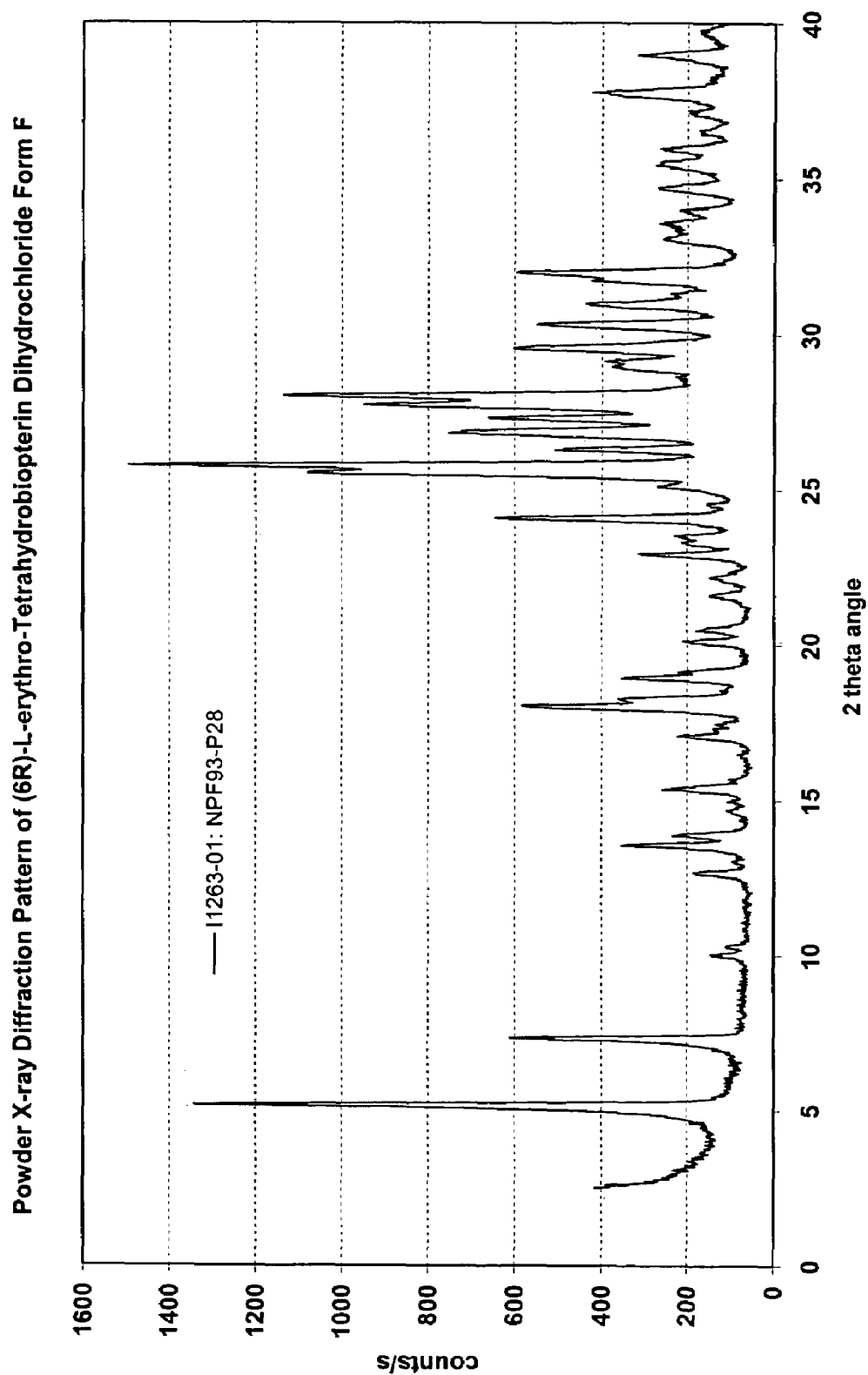


Figure 3

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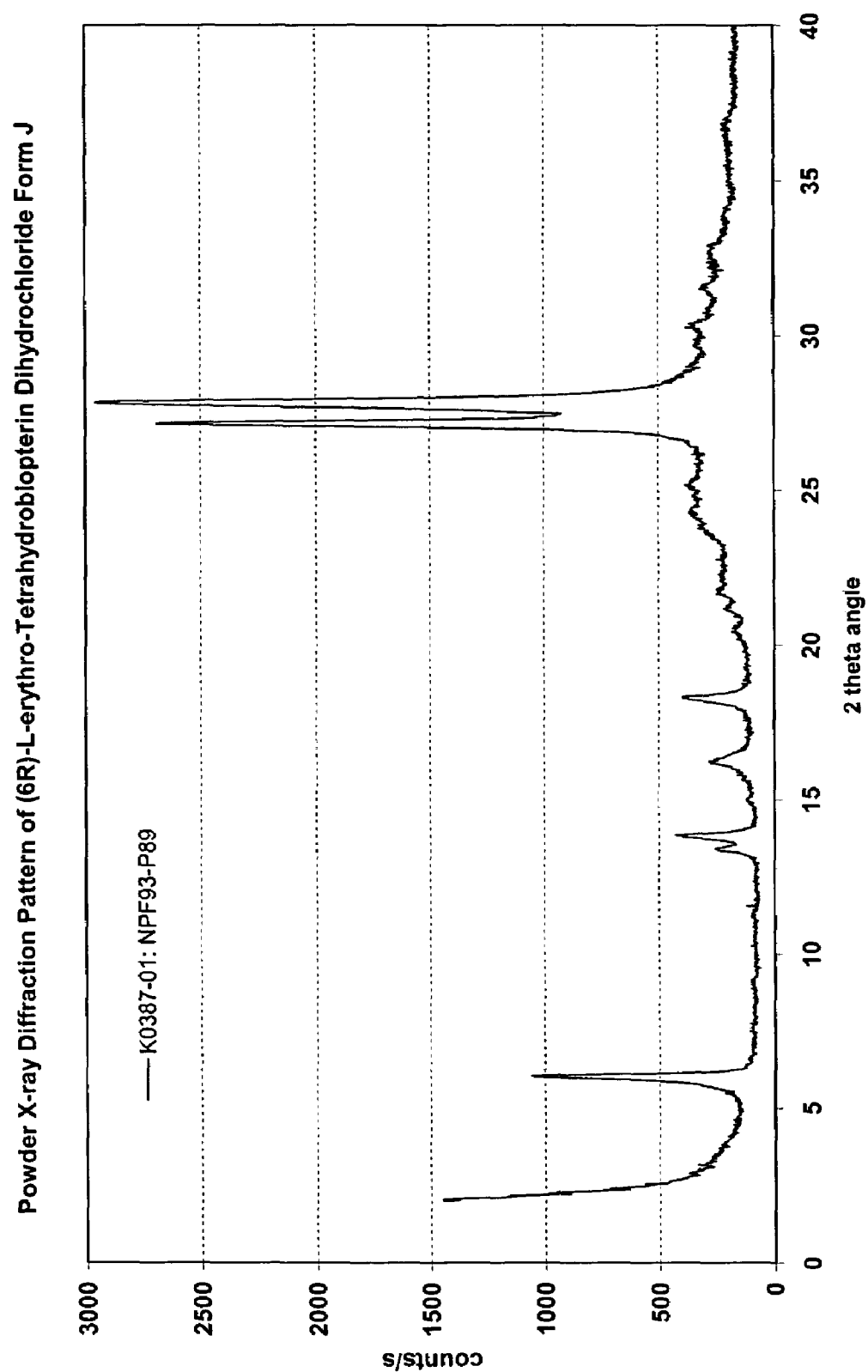


Figure 4

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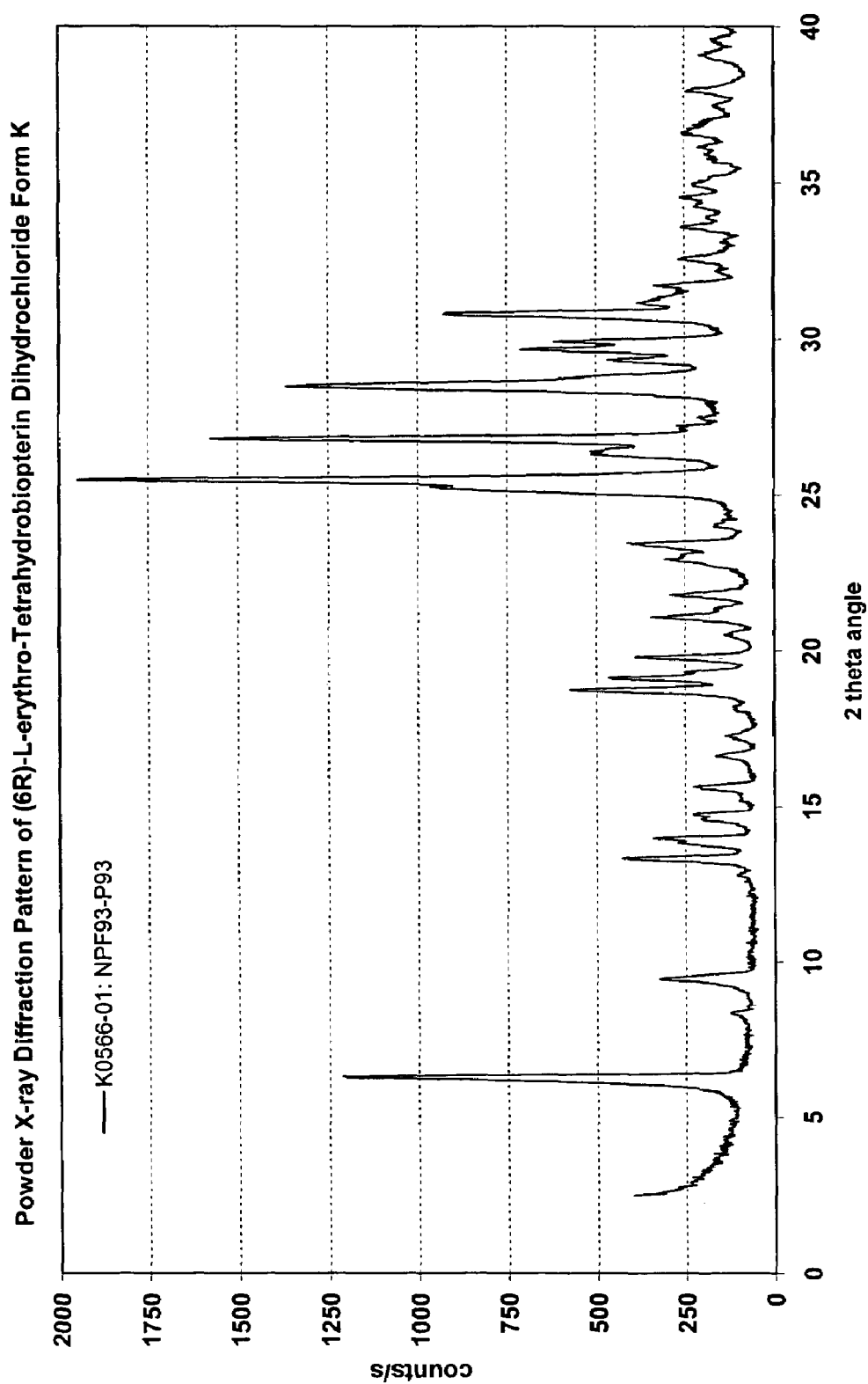


Figure 5

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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochlorid Form C

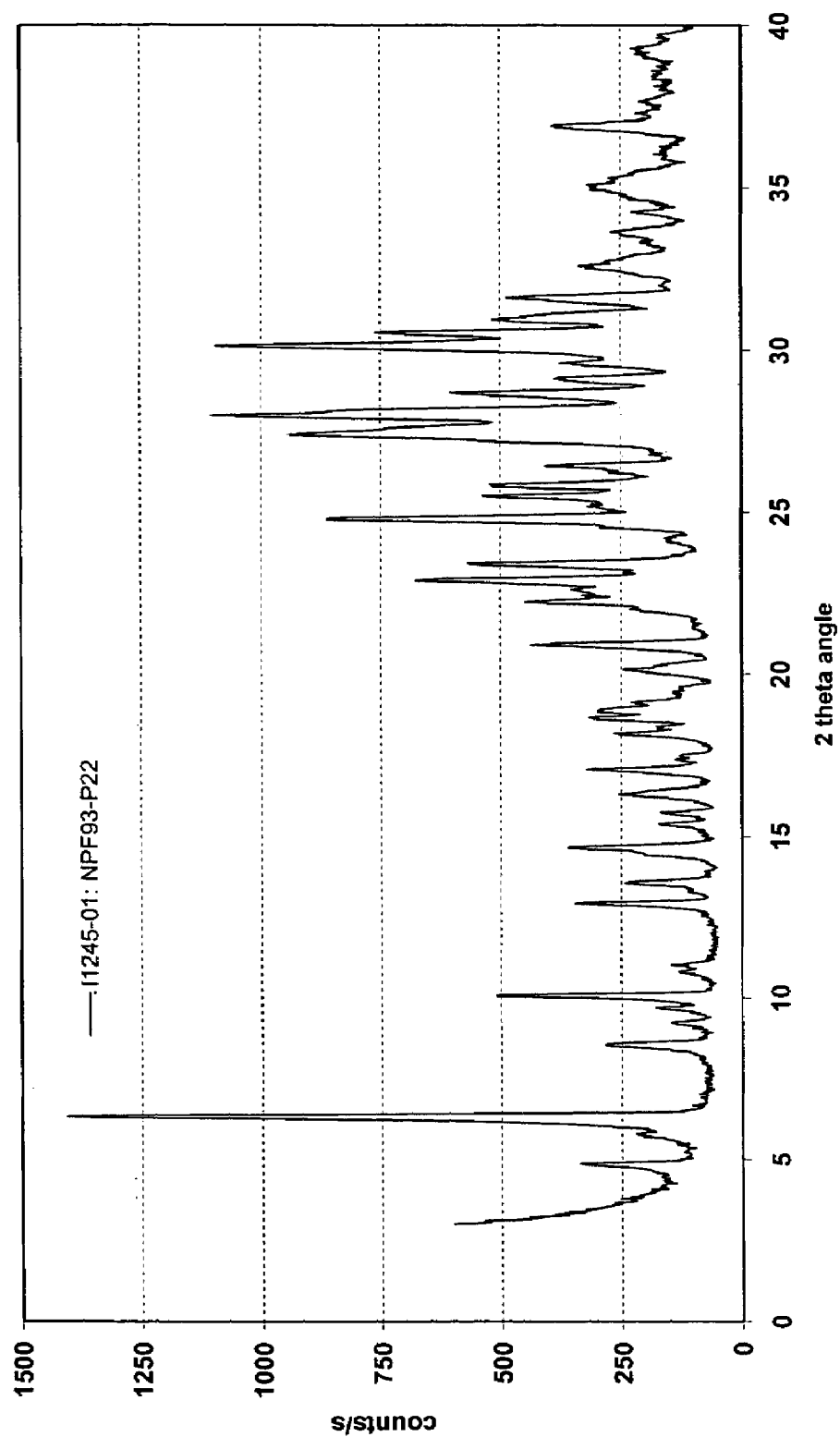


Figure 6

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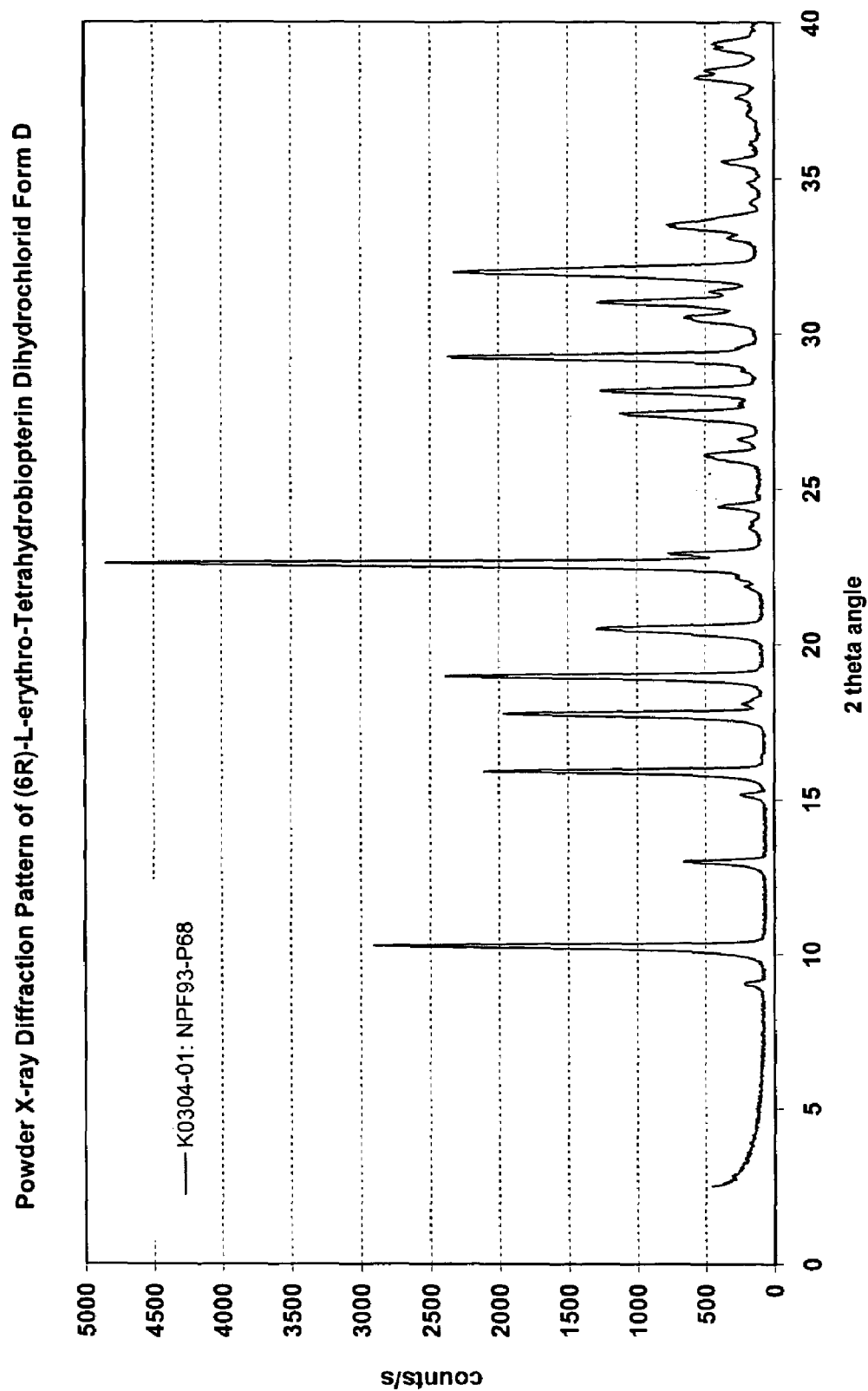


Figure 7



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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form E

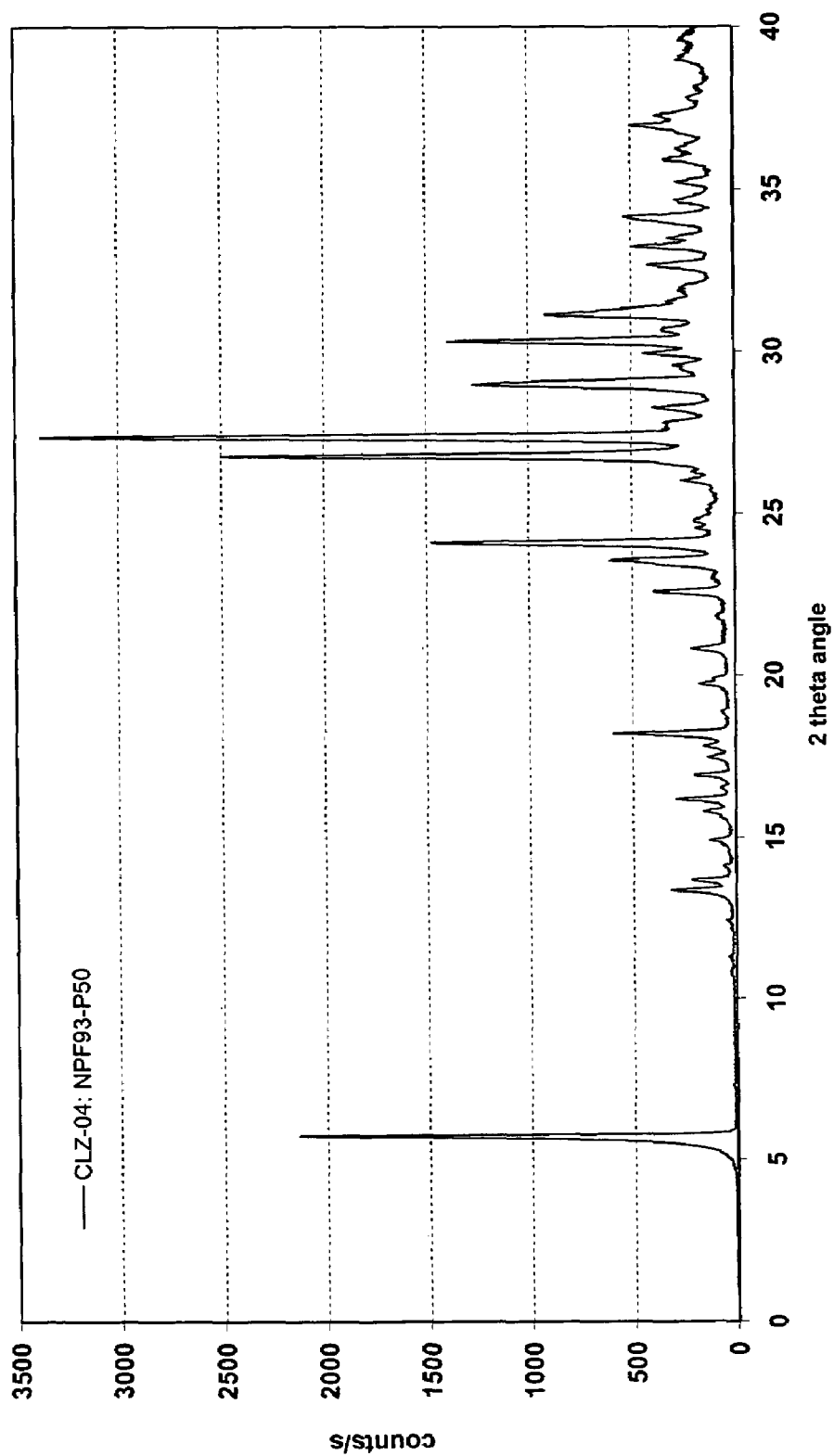


Figure 8

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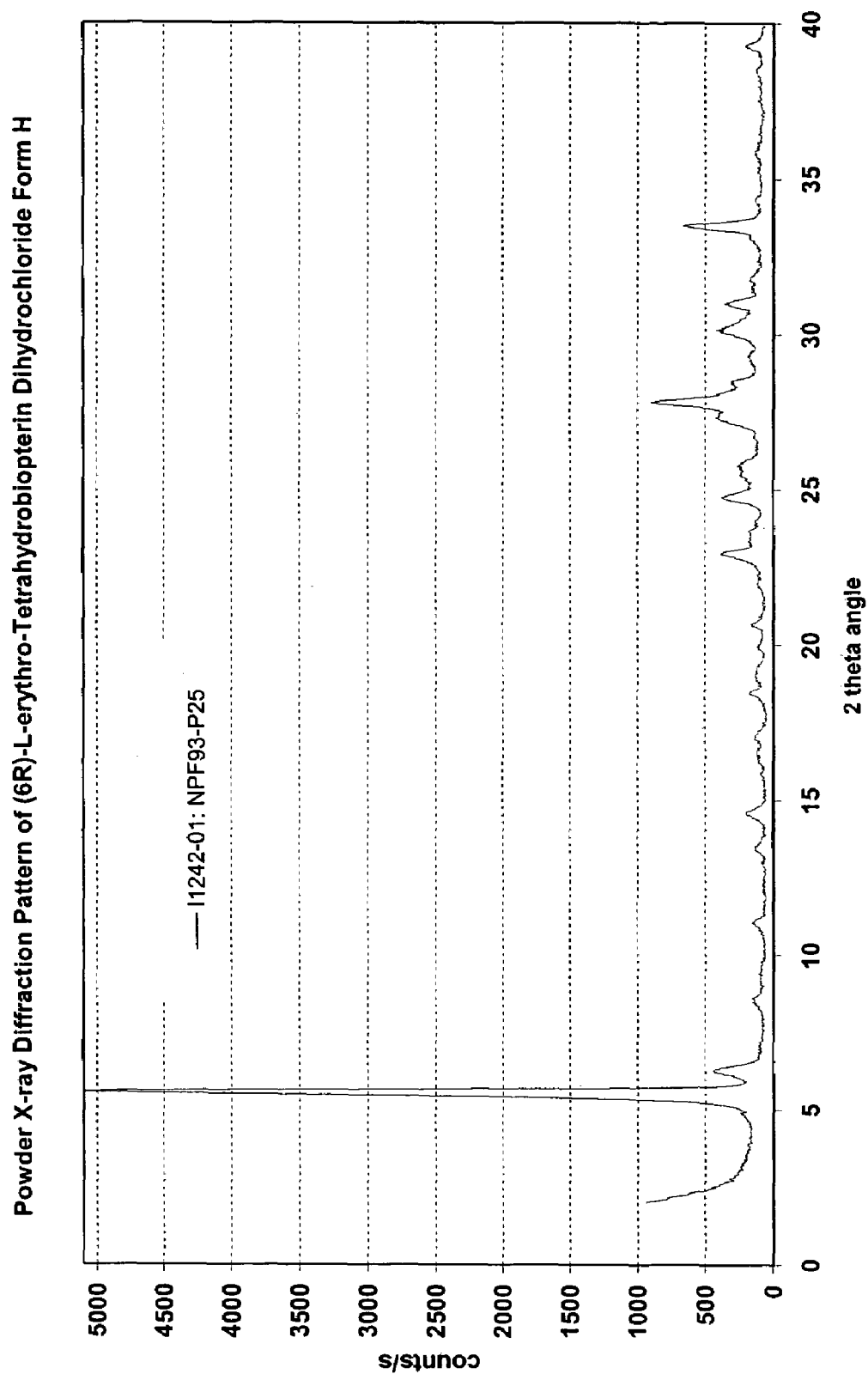


Figure 9

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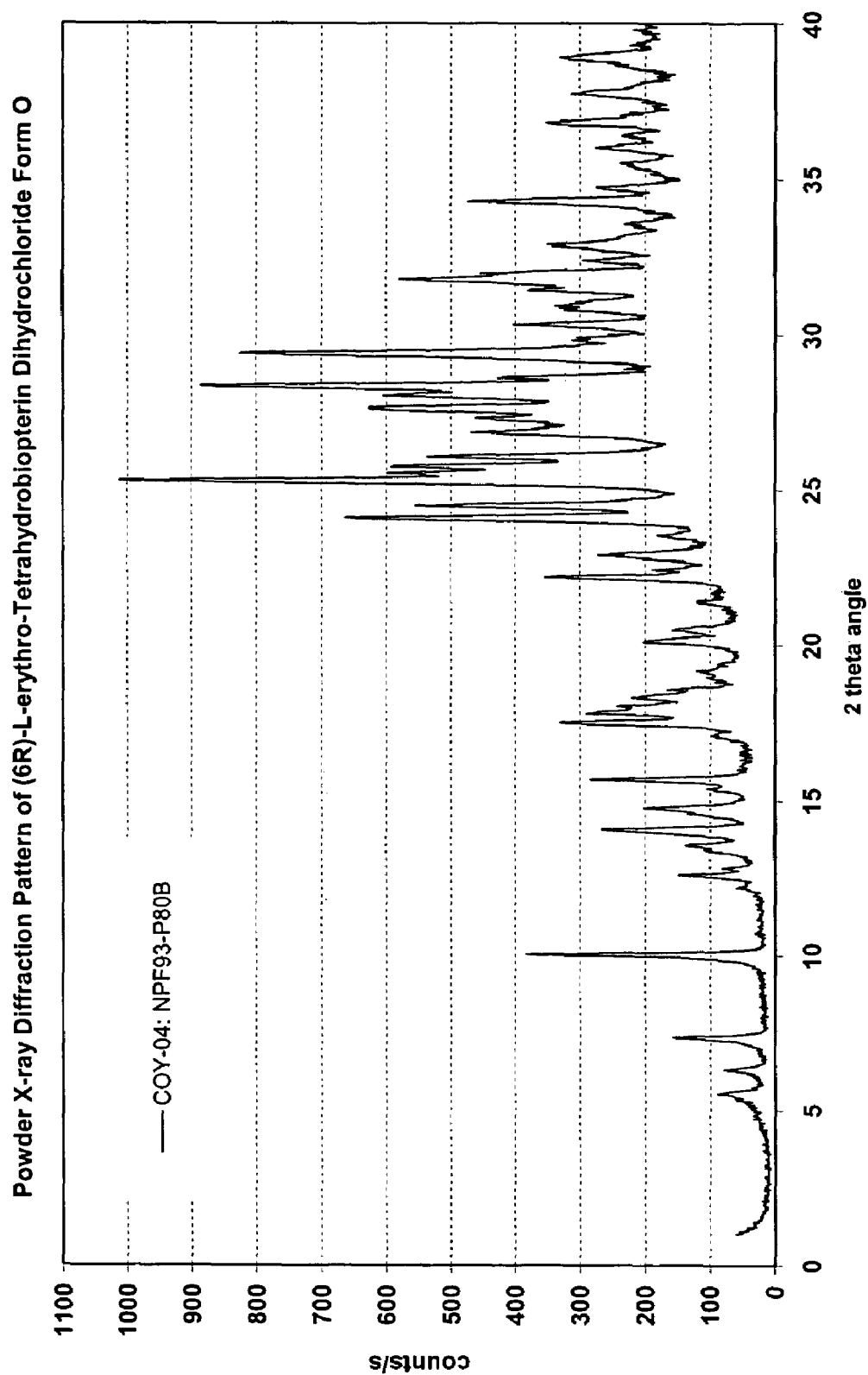


Figure 10

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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form G

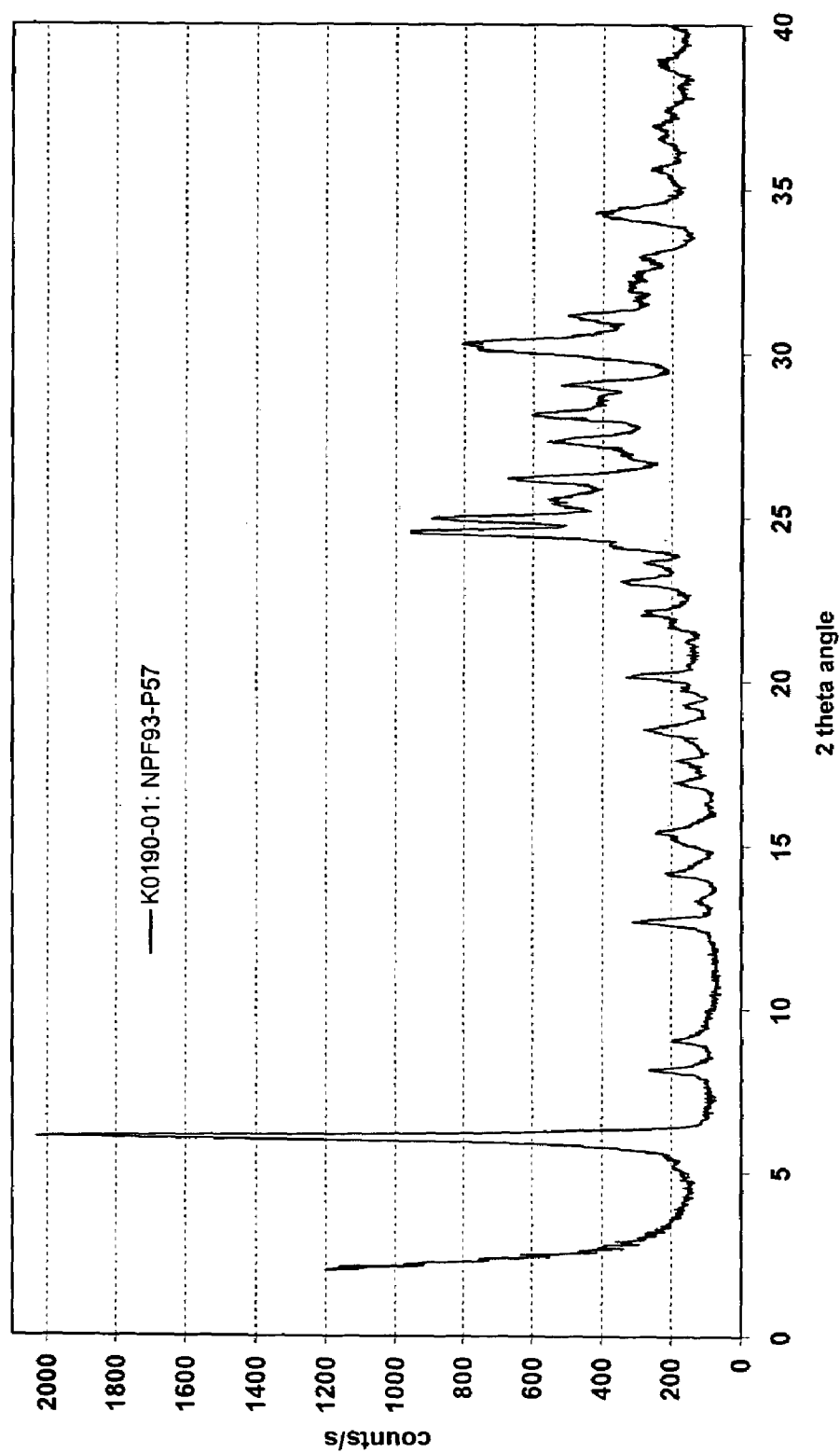


Figure 11

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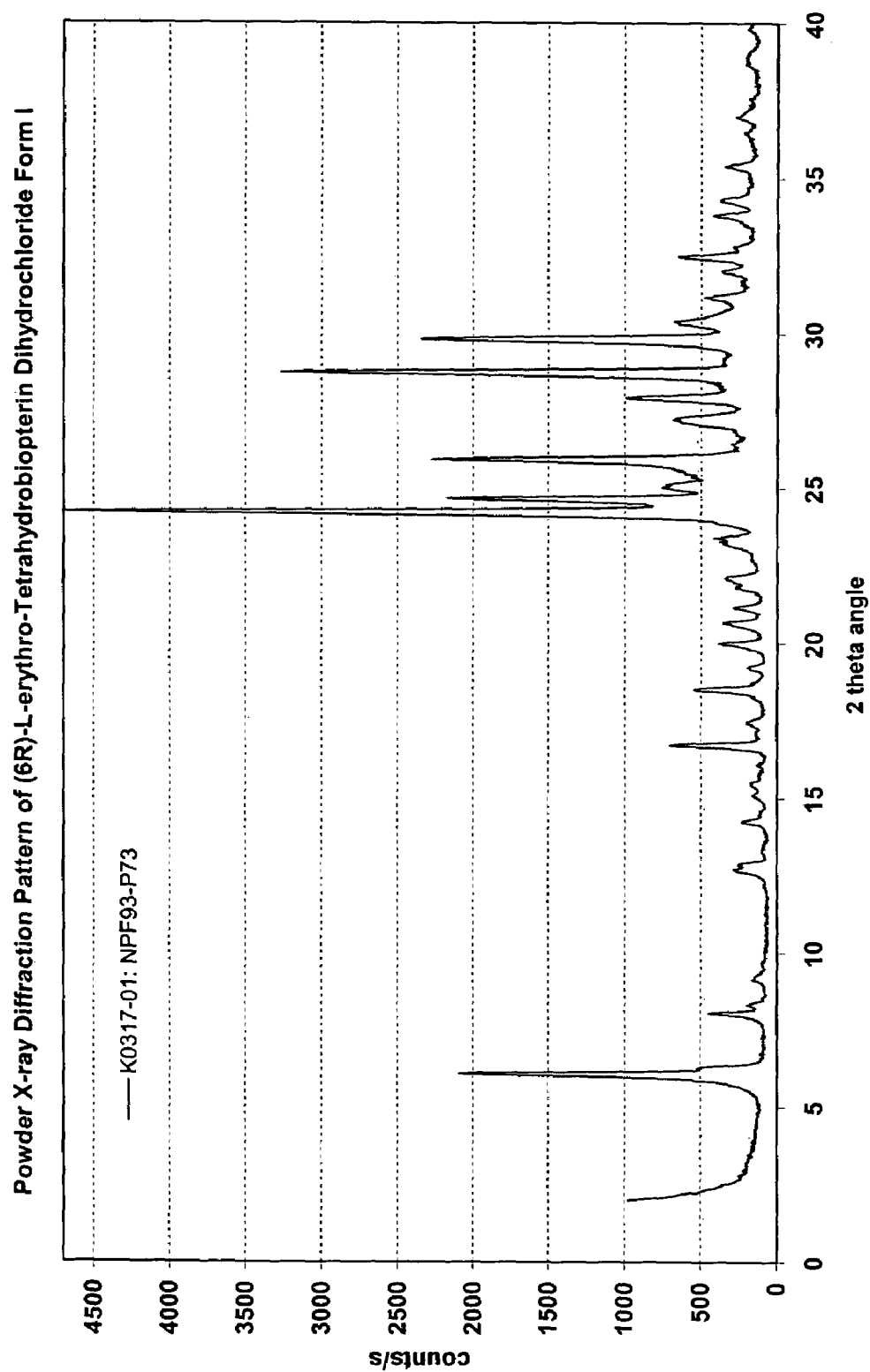


Figure 12

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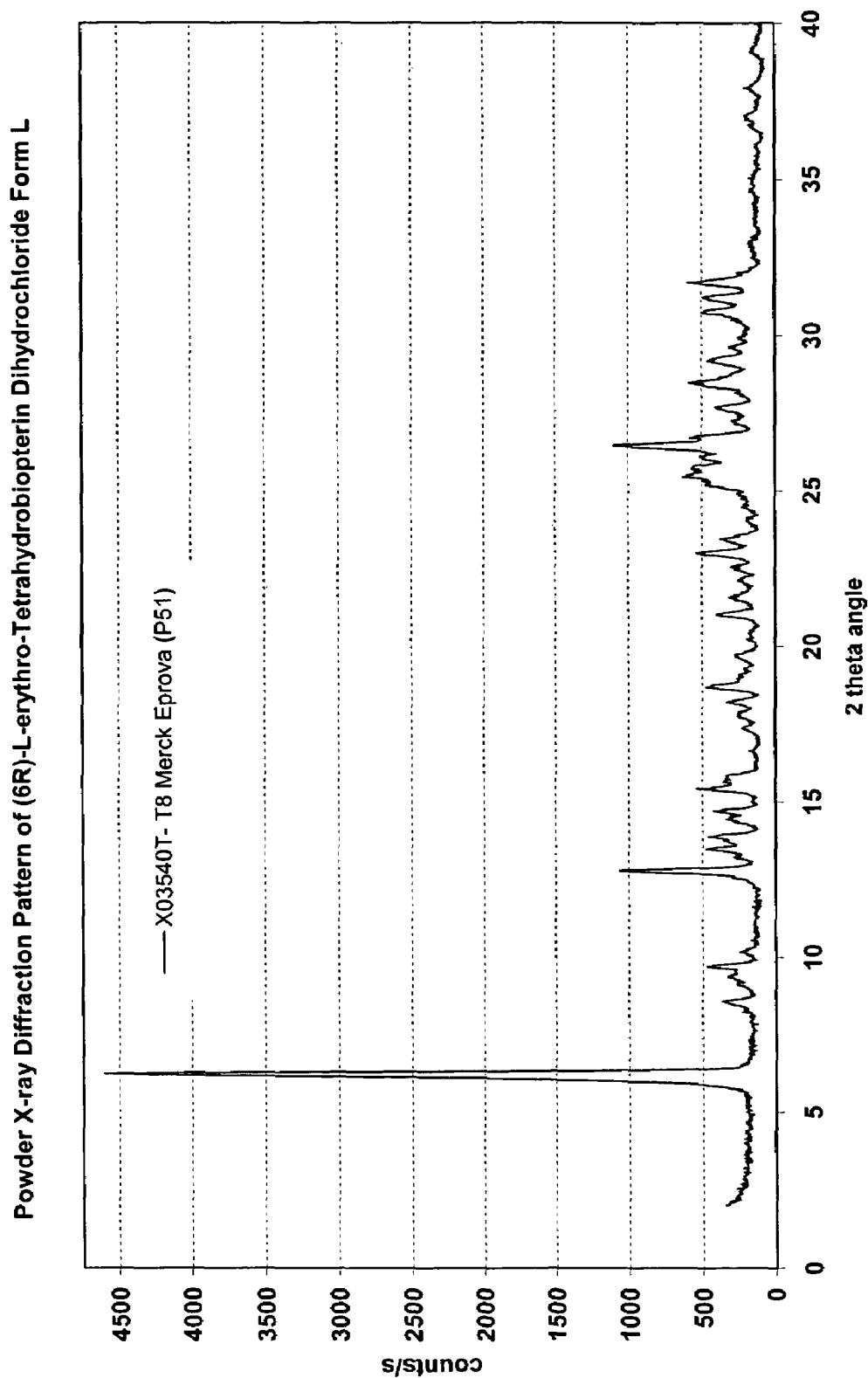


Figure 13

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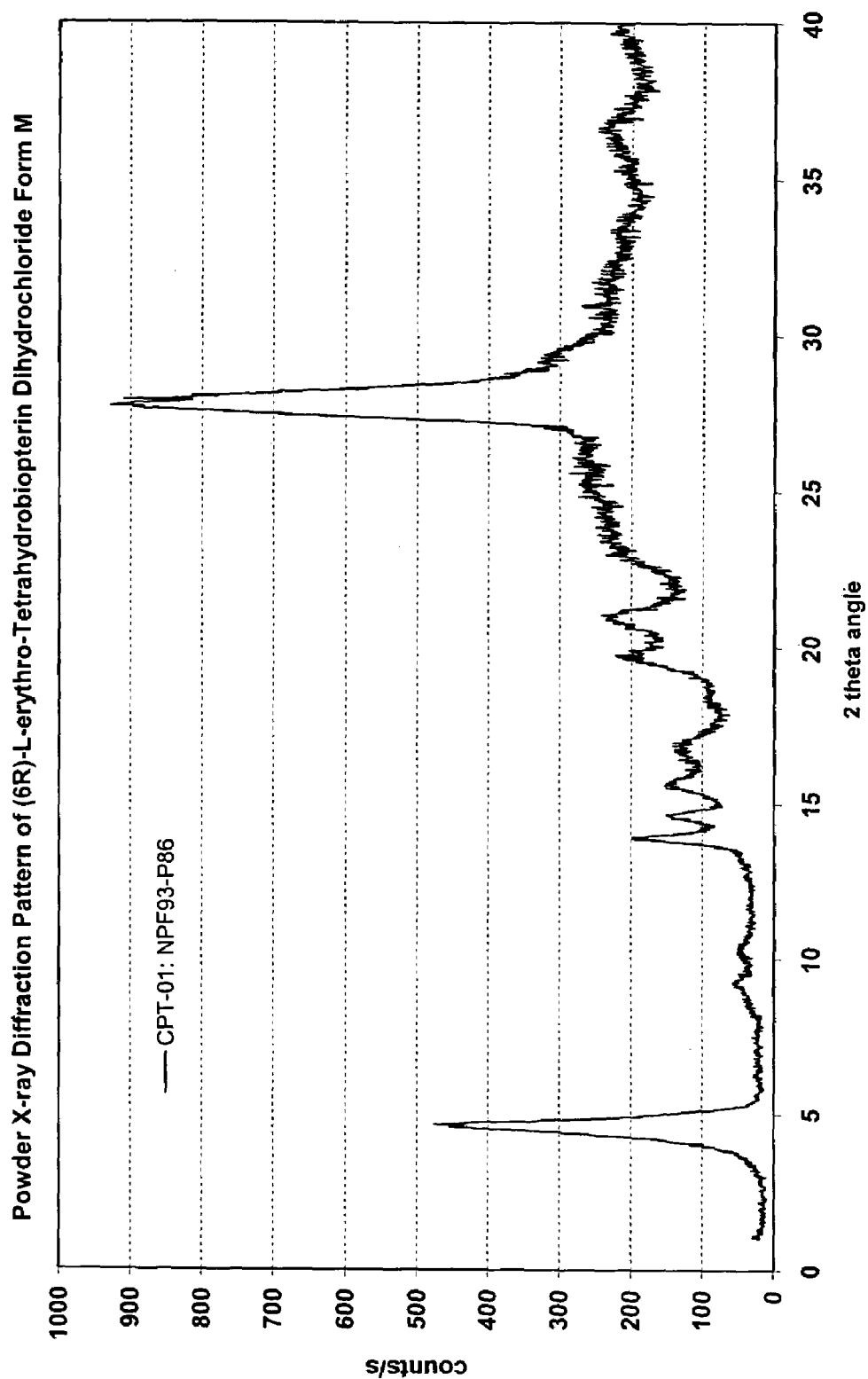


Figure 14

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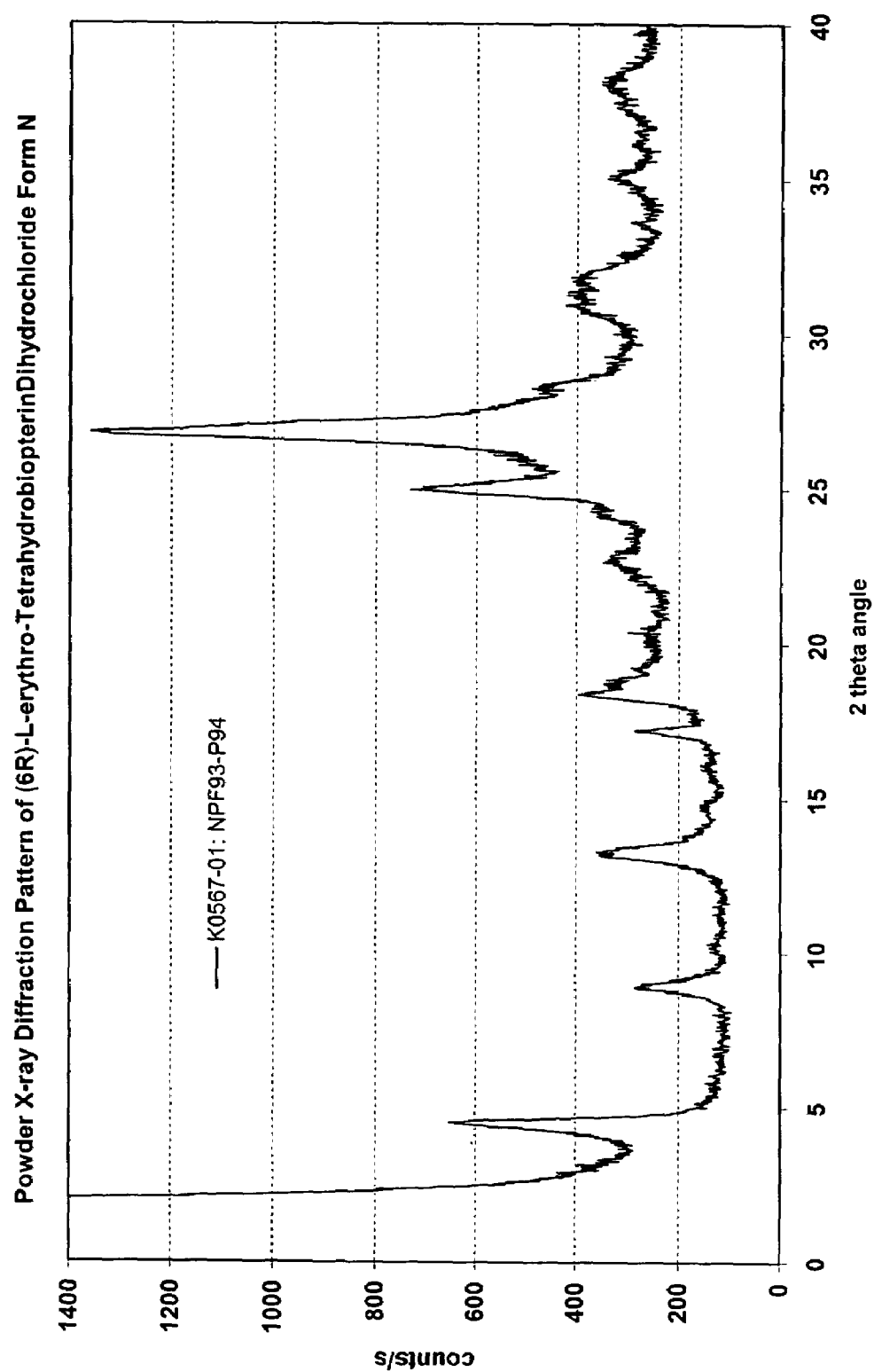


Figure 15



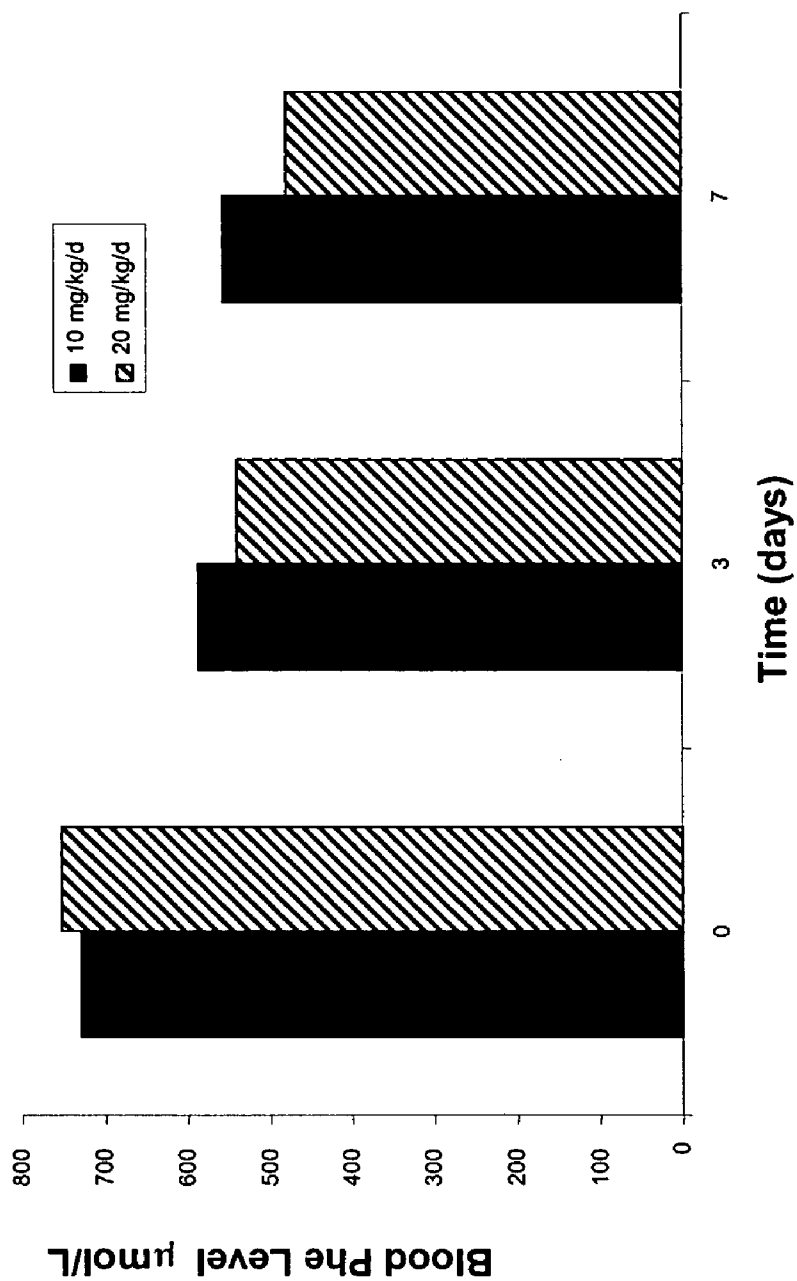
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**Mean Blood Phe Level 3 and 7 Days After  
Multiple Daily BH4 Doses of 10 and 20  
mg/kg in PKU Patients (N=20)**



**Figure 16**

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# Individual Blood Phe in 12 Adults with PKU on 10 mg/kg BH4 Daily

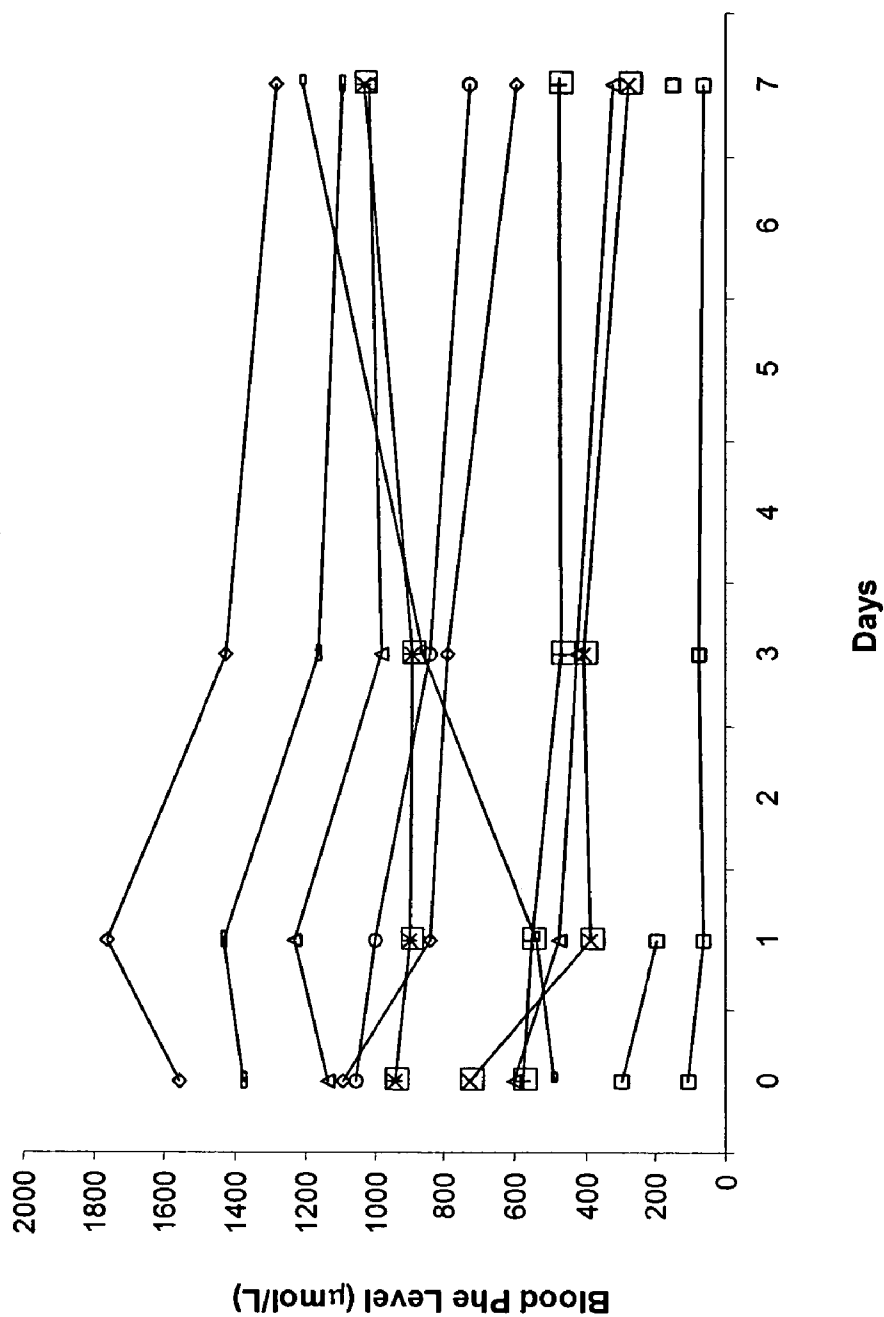


Figure 17

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# Individual Blood Phe in 12 Adults with PKU on 20 mg/kg BH4 Daily

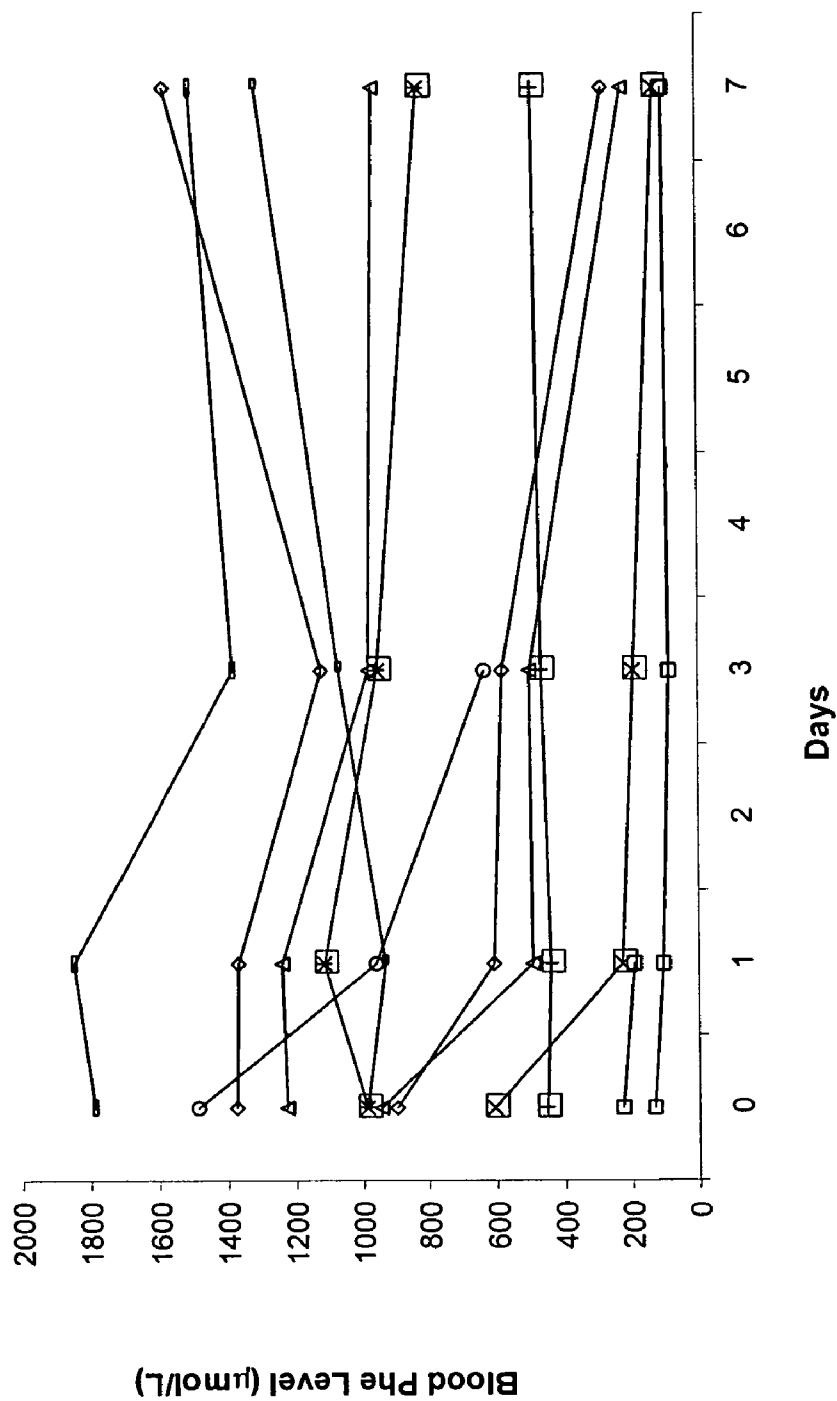


Figure 18

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# Individual Blood Phe in 8 Children with PKU on 10 mg/kg BH4 Daily

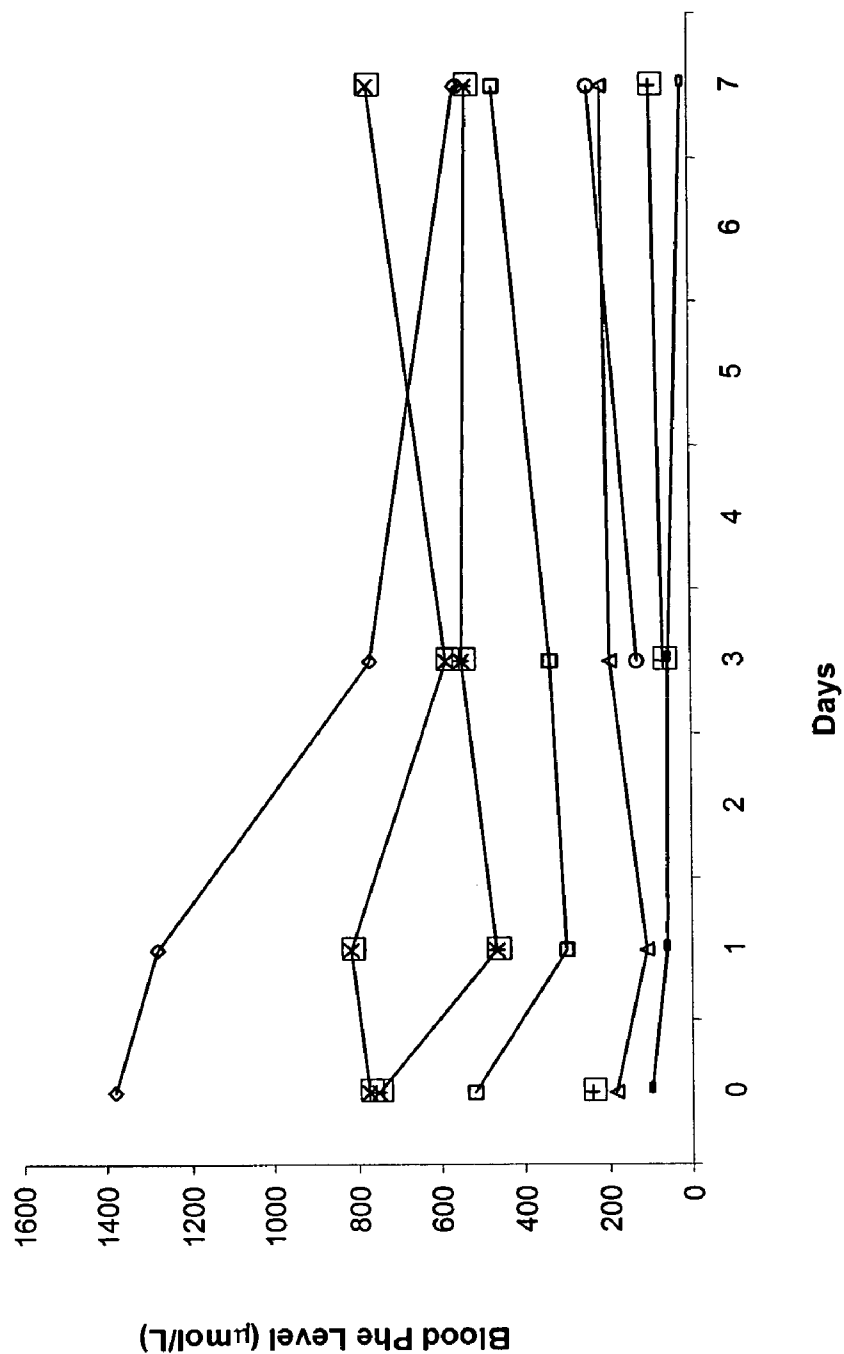


Figure 19

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# Individual Blood Phe in 8 Children with PKU on 20 mg/kg BH4 Daily

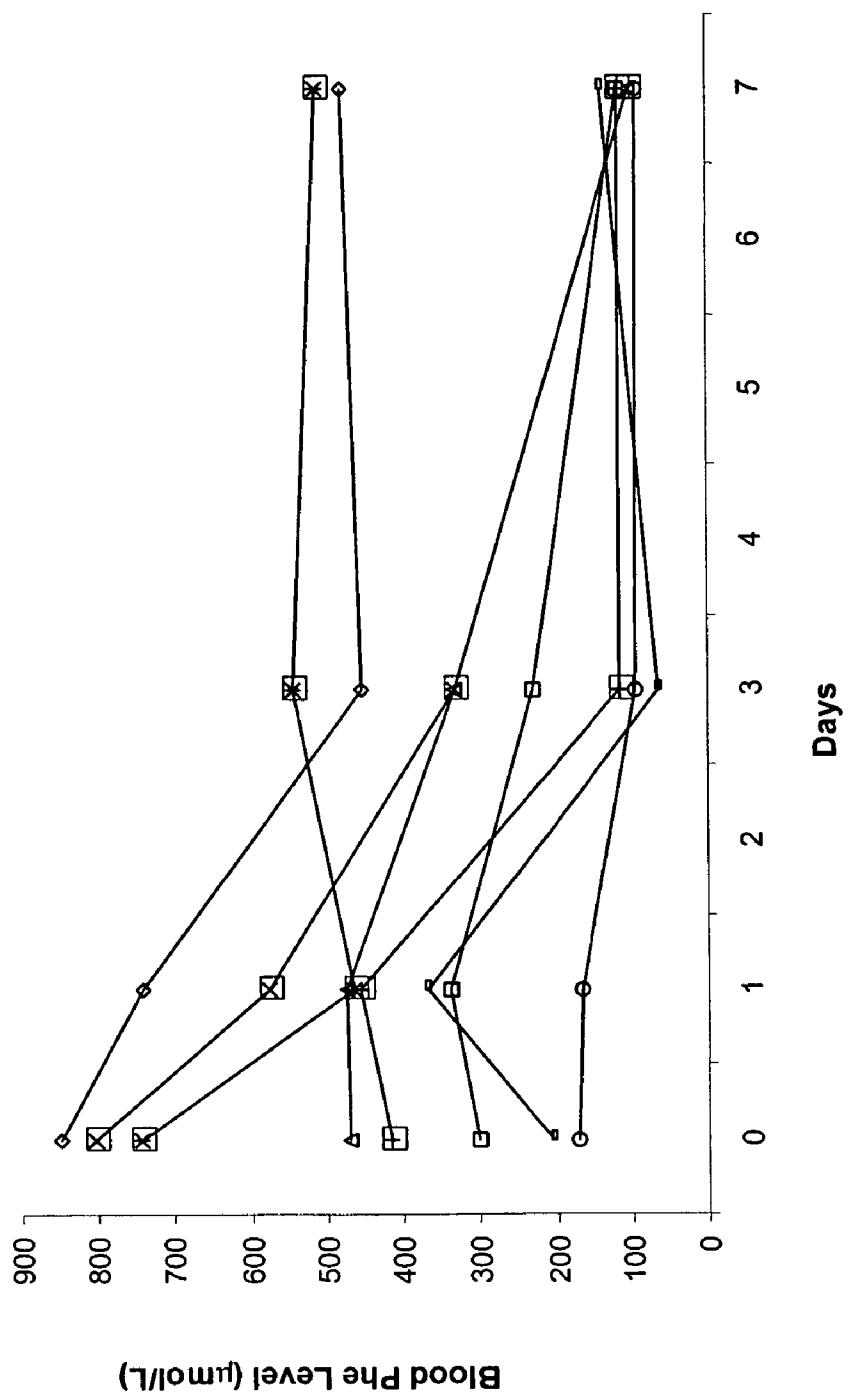


Figure 20

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## METHODS AND COMPOSITIONS FOR THE TREATMENT OF METABOLIC DISORDERS

### CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of U.S. patent application Ser. No. 10/991,573, which was filed on Nov. 17, 2004 and which claimed the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Ser. No. 60/520,767, which was filed Nov. 17, 2003. The entire disclosure of each of these priority applications is hereby incorporated herein by reference.

### BACKGROUND

#### 1. Field

The present invention is generally directed to the therapeutic intervention of metabolic disorders, particularly those involving amino acid metabolism. More particularly, the present invention is directed to methods and compositions for the treatment of phenylketonuria, vascular diseases, ischemic or inflammatory diseases, or insulin resistance, or conditions and patients that would benefit from enhancement of nitric oxide synthase activity.

#### 2. Background of the Related Technology

Phenylketonuria (PKU) is an inherited metabolic disorder that was first identified in the 1930s. In most cases, and until the mid-1990s, it was thought that this is a disorder of amino acid metabolism resulting from a deficiency in the liver enzyme phenylalanine hydroxylase (PAH). Deficiencies in PAH in turn result in an excess of phenylalanine (Phe) in the brain and plasma. The deficiency in PAH ultimately manifests in a lack of tyrosine, which is a precursor for the neurotransmitters.

Left undetected and untreated early in the life of an infant, PKU leads to irreversible damage of the nervous system, severe mental retardation and poor brain development. Features other than mental retardation in untreated patients include brain calcification, light pigmentation, peculiarities of gait, stance, and sitting posture, eczema, and epilepsy. It has been reported that an infant suffers a loss of 50 IQ points within the first year of infancy and PKU is invariably accompanied by at least some loss of IQ. Once detected, the condition is treated by providing the infant, and later the child, with a low Phe diet. In adults, the protein supplements routinely taken by classic PKU patients may be Phe-free with the assumption that such adults will receive sufficient quantities of Phe through the remaining diet, controlled under a strict regimen, so that the overall diet is a low Phe diet. Also, pregnant women who suffer from the condition are recommended a diet that is low in Phe to avoid the risk of impairment of the development of the fetus and congenital malformation (maternal PKU syndrome).

In more recent years it has been shown that pathological symptoms which manifest from the condition of excess of Phe, collectively termed hyperphenylalaninemia (HPA), may be divided into multiple discrete disorders, which are diagnosed according to plasma Phe concentrations and responsiveness to a co-factor for PAH. At an initial level, HPAs may be divided into HPA caused as a result of a deficiency in the cofactor 6R-L-erythro-5,6,7,8, tetrahydrobiopterin (BH4; malignant PKU) and HPA resulting from a deficiency in PAH. The latter category is further subdivided into at least three categories depending on the plasma concentration of Phe in the absence of dietary or other therapeutic intervention (referred to herein as "unrestricted plasma Phe concentration").

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Normal plasma Phe homeostasis is tightly controlled resulting in a plasma Phe concentration of  $60 \mu\text{mol/L} \pm 15 \mu\text{mol/L}$ . Classical PKU (OMIM No. 261600) is the most severe form of PKU and it results from null or severe mutations in PAH, which lead to unrestricted plasma Phe concentrations greater than  $1200 \mu\text{mol/L}$  when left untreated. Individuals with classical (or severe) PKU must be treated with a strict dietary regimen that is based on a very low Phe diet in order to reduce their Phe concentrations to a safe range. Milder forms of HPA also have been characterized. A less severe form of PKU is one which manifests in plasma Phe concentrations of  $10\text{--}20 \text{ mg/dL}$  ( $600\text{--}1200 \mu\text{mol/L}$ ), and is generally termed "mild PKU". This moderate form of PKU is managed through the use of moderate dietary restrictions, e.g., a low total protein diet, but otherwise not necessarily Phe-free. Finally, mild HPA, also referred to as benign or non-PKU HPA is characterized by plasma Phe concentrations of between  $180\text{--}600 \mu\text{mol/L}$ . The individuals with non-PKU HPA are not routinely treated as they are considered to have plasma Phe levels that are within the "safe" range. Nevertheless, as mentioned above, these Phe levels are still significantly elevated in these individuals as compared to normal, non-PKU subjects and may present detrimental sequelae in at least pregnant women and very young patients. For a more detailed review of HPA resulting from PAH deficiency, those of skill in the art are referred to Scriver et al., 2001 (*Hyperphenylalaninemia: Phenylalanine Hydroxylase Deficiency*, In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. The Metabolic and Molecular Bases of Inherited Disease. 8th ed. New York: McGraw-Hill, 2001: 1667-1724). NIH Guidelines indicate that for children with PKU, it is preferable reduce the plasma Phe to be  $360\text{--}420 \mu\text{mol/L}$ .

HPA also results from defects in BH4 metabolism. BH4 is an essential cofactor of both tyrosine and tryptophan hydroxylase, the rate limiting enzymes in the biosynthesis of the neurotransmitters dopamine and serotonin. The effects of deficiencies in dopamine and serotonin are collectively known as "atypical" or "malignant" HPA. Thus, traditional diagnoses of HPA have involved a determination of whether the HPA is a result of BH4 deficiency or PAH deficiency. Typically, diagnosis of PKU is established on the basis of a persistently elevated blood Phe concentration. Following a positive screen for elevated blood Phe (plasma Phe  $>120 \mu\text{mol/L}$ ; Weglage et al., *J. Inherit. Metab. Dis.*, 25:321-322, 2002), a differential screen is performed in which it is determined whether the elevated Phe is a result of BH4 deficiency or PAH deficiency. The differential diagnosis involves determining whether the elevated Phe concentration is decreased as a result of BH4 administration (BH4 loading test). The BH4 loading test typically involves a one-time load of BH4 e.g.,  $5\text{--}20 \text{ mg/kg}$  being administered to the subject who is on a normal (i.e., unrestricted) diet and determining whether the subject experiences a decrease in Phe levels (see e.g., Ponzzone et al., *Eur. J. Pediatr.* 152:655-661, 1993; Weglage et al., *J. Inherit. Metab. Dis.*, 25:321-322, 2002.)

Typically, individuals that respond to a BH4 loading test by a decrease in plasma Phe levels are diagnosed as having a defect in BH4 homeostasis. However, there have been various reports of patients with a BH4 responsive type of PAH deficiency (Kure et al., *J. Pediatr.* 135:375-378, 1999; Lasker et al., *J. Inherit. Metab. Dis.* 25:65-70, 2002; Linder et al., *Mol. Genet. Metab.* 73:104-106, 2001; Spaapen et al., *Mol. Genet. and Metabolism*, 78:93-99, 2003; Trefz et al., 2001). These subjects have plasma Phe levels that are typical of moderate PKU, i.e., less than  $1000 \mu\text{mol/L}$  and typically less than  $600 \mu\text{mol/L}$ . Patients that have severe classical PKU are



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not responsive to typical 24 hour BH4 loading tests (Ponzone et al., *N. Engl. J. Med* 348(17):1722-1723, 2003).

It has been suggested that individuals that are responsive to BH4 do not require dietary intervention, but rather should be treated with BH4. Likewise, the converse has been suggested for subjects that have been diagnosed as non-responsive to the BH4 loading test, i.e., these subjects should be treated with dietary restriction and not BH4 therapy. Ponzone et al. particularly noted that individuals that have severe phenylketonuria will not respond to BH4 therapy and such therapy should not be used on these patients (Ponzone et al., *N. Engl. J. Med* 348(17):1722-1723, 2003). Thus, presently there are divergent therapeutic regimens for treatment of HPA depending on whether or not the individual is responsive to BH4. Moreover, it has been suggested that very few patients will benefit from BH4 therapy. In fact, it is thought that the only individuals with a PAH-deficient form of HPA that will benefit from BH4 therapy are those with mild PKU. As these individuals will typically have Phe levels in the safe range (i.e., less than 600  $\mu\text{M}$ ), the disease state can be controlled using moderate dietary restriction (see Hanley, *N. Engl. J. Med* 348(17):1723, 2003). Thus, BH4 therapy either alone, or in combination with any other therapeutic intervention, has not been considered as a viable therapeutic intervention for the vast majority of individuals with HPA.

BH4 is a biogenic amine of the naturally-occurring pterin family. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. This is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (*Disorders of tetrahydrobiopterin and related biogenic amines*. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1275-1776). Despite the elucidation of the role of BH4 deficiency in HPA, treatment with BH4 has not been suggested because such treatment is very expensive, as high as \$30,000 per year for an adolescent or adult, as compared with \$6,000 for phenylalanine-restricted dietary therapy (Hanley, *N. Engl. J. Med* 348(17):1723, 2003). Another significant problem with BH4 is that this compound is unstable and readily undergoes aerobic oxidation at room temperature (Davis et al., *Eur. J. Biochem.*, Vol 173, 345-351, 1988; U.S. Pat. No. 4,701,455) and has a shelf-life of less 8 hours at room temperature (Bernegger and Blau, *Mol. Genet. Metabol.* 77:304-313, 2002).

Thus, to date, dietary intervention is the typical therapeutic intervention used for all patients with severe classical PKU and in many patients with moderate PKU. Such dietary intervention typically entails restricting the patient to foodstuff that is composed of natural foods which are free from, or low in, Phe. However, in addition to eliminating Phe, such a dietary regimen eliminates many sources of other essential amino acids, vitamins and minerals. Consequently, without supplementation, such a diet provides inadequate protein, energy, vitamins and minerals to support normal growth and development. As PKU is a manifestation of a lack of tyrosine, which arises due to the lack of hydroxylation of phenylalanine, tyrosine becomes an essential amino acid and dietary supplements for PKU must contain a tyrosine supplement. Therefore, it is common to use nutritional formulas to supplement the diets of PKU patients. Also, for babies, it is common to use infant formulas which have a low Phe content as the sole or primary food source.

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However, dietary protein restriction is at best an ineffective way of controlling PKU in many classes of patients. For example, treatment is of paramount importance during pregnancy because high Phe levels may result in intrauterine retardation of brain development. However, a low protein diet during pregnancy may result in retarded renal development and is thought to produce a subsequent reduction in the number of nephrons and potentially leads to hypertension in adulthood. (D'Agostino, *N. Engl. J. Med.* 348(17)1723-1724, 2003).

Poor patient compliance with a protein-restricted diet also is a problem. The Phe-free protein formulae available are bitter tasting making it difficult to ensure that the patient consumes sufficient quantities of the protein to maintain the required daily intakes of protein, amino acids, vitamins, minerals, and the like. This is particularly a problem with older children who may require up to 70 g, dry weight, of the formulas per day. For example, Schuett, V. E.; 1990; DHHS Publication No HRS-MCH-89-5, reports that more than 40% of PKU patients in the US of eight years or older no longer adhere to the dietary treatment. (U.S. Pat. No. 6,506,422). Many adolescent patients fail to rigorously follow the protein-restricted diet due to fears of peer attitude.

Thus, there remains a need for a therapeutic medicament to replace or supplement and alleviate the dietary restrictions under which a PKU patient is placed. The present invention is directed to addressing such a need.

#### SUMMARY OF THE INVENTION

The invention describes intervention in metabolic disorders, particularly those involving amino acid metabolism. More particularly, the present invention is directed to methods and compositions for the treatment of subjects exhibiting elevated phenylalanine levels, for example, subjects suffering from hyperphenylalanemia, mild phenylketonuria or classic severe phenylketonuria; and methods and compositions for the treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity; and methods and compositions for treatment of subjects suffering from vascular diseases, ischemic or inflammatory diseases, diabetes, or insulin resistance.

In one aspect, the invention describes methods of treating classic severe phenylketonuria (PKU) in a subject comprising administering to the subject a protein-restricted diet in combination with a composition comprising tetrahydrobiopterin (BH4) or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of the combined administration. In specific embodiments, the subject is one who does not manifest a deficiency in BH4 homeostasis. The subject may be an individual that does not manifest symptoms of L-dopa neurotransmitter deficiency.

A subject selected from treatment according to the methods of the invention will have an elevated plasma Phe concentration, such a concentration may be greater than 1800  $\mu\text{M/L}$  in the absence of the therapeutic. Other embodiments contemplate that has a plasma phenylalanine concentration of greater than 1000  $\mu\text{M}$  in the absence of a therapeutic regimen. In preferred embodiments, the combined administration methods of the invention decrease the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ . More preferably, it is decreased to less than 500  $\mu\text{M}$ . Even more preferably, the combined administration decreases the plasma phenylalanine concentration of the subject to 360  $\mu\text{M} \pm 15 \mu\text{M}$ .

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The BH4 is preferably administered in an amount of between about 1 mg/kg to about 30 mg/kg, more preferably between about 5 mg/kg to about 30 mg/kg. The BH4 may be administered in a single daily dose or in multiple doses on a daily basis. In some embodiments, the BH4 therapy is not continuous, but rather BH4 is administered on a daily basis until the plasma phenylalanine concentration of the subject is decreased to less than 360  $\mu$ M. Preferably, wherein the plasma phenylalanine concentration of the subject is monitored on a daily basis and the BH4 is administered when a 10% increase in plasma phenylalanine concentration is observed. Preferably, the BH4 being administered is a stabilized crystallized form of BH4 that has greater stability than non-crystallized stabilized BH4. More preferably, the stabilized crystallized form of BH4 comprises at least 99.5% pure 6R BH4. Precursors such as dihydrobiopterin (BH2), and sepiapterin also may be administered. BH4 may be administered orally

The protein-restricted diet administered in the methods herein is one that is a phenylalanine-restricted diet wherein the total phenylalanine intake of the subject is restricted to less than 600 mg per day. In other embodiments, the protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 300 mg per day. In still other embodiments, the protein-restricted diet is one which is supplemented with amino acids, such as tyrosine, valine, isoleucine and leucine. In certain embodiments, protein-restricted diet comprises a protein supplement and the BH4 is provided in the same composition as the protein supplement.

In specific embodiments, the subject is one which has been diagnosed as having a mutant phenylalanine hydroxylase (PAH). The mutant PAH may comprise a mutation in the catalytic domain of PAH. Exemplary such mutations include one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C.

Also contemplated herein is a method for the treating a pregnant female having hyperphenylalaninemia (HPA) comprising administering to the subject a protein-restricted diet in combination with a composition comprising tetrahydrobiopterin (BH4) or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of the combined administration. In certain embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 180  $\mu$ M but less than 600  $\mu$ M. In other embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 500  $\mu$ M but less than 1200  $\mu$ M. In still other embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 1000  $\mu$ M.

Also contemplated is a method of treating a patient having above normal concentration of plasma phenylalanine (e.g., greater than 180  $\mu$ M/L and more preferably, greater than 360  $\mu$ M/L) comprising administering to the patient a stabilized BH4 composition in an amount effective to produce a decrease in the plasma phenylalanine concentration of the patient. Preferably, the stabilized BH4 composition is stable at room temperature for more than 8 hours. The patient will likely have a plasma phenylalanine concentration greater than 180  $\mu$ M prior to administration of the BH4. More particularly, the patient has a plasma phenylalanine concentration of between 120  $\mu$ M and 200  $\mu$ M. In other embodiments, the patient has a plasma phenylalanine concentration of between

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200  $\mu$ M and 600  $\mu$ M. In still other embodiments, the patient has a plasma phenylalanine concentration of between 600  $\mu$ M and 1200  $\mu$ M. Yet another class of patients to be treated are those that have an unrestricted plasma phenylalanine concentration greater than 1200  $\mu$ M. In specific embodiments, the patient is an infant, more particularly, an infant having a plasma phenylalanine concentration greater than 1200  $\mu$ M. In other embodiments, the patient is pregnant and pregnant patient has a plasma phenylalanine concentration of between about 200  $\mu$ M to about 600  $\mu$ M. Pregnant patients with a plasma phenylalanine concentration greater than 1200  $\mu$ M are particularly attractive candidates for this type of therapy, as are patient who are females of child-bearing age that are contemplating pregnancy. In those embodiments, in which the patient has a plasma phenylalanine concentration greater than 1200  $\mu$ M, and the method further comprises administering a protein-restricted diet to the patient.

The invention also contemplates a method of treating a patient having phenylketonuria, comprising administering to the patient a stabilized BH4 composition in an amount effective to produce a decrease in the plasma phenylalanine concentration of the patient wherein the patient has been diagnosed as unresponsive to a single-dose BH4 loading test. Preferably, the patient is unresponsive within 24 hours of the BH4 load.

Another related aspect of the invention provides a multiple dose loading test that involves administration of more than one dose of BH4. The data described herein demonstrates that subjects who are considered "unresponsive" to a single dose BH4 loading test can respond to multiple doses of BH4 with a significant reduction in phenylalanine levels. In one embodiment, at least two doses of BH4 which may be between about 5 mg to 40 mg are administered to a subject over a time period of more than one day, preferably 7 days.

The treatment methods according to the invention may comprise administering between about 10 mg BH4/kg body weight to about 200 mg BH4/kg body weight. The BH4 may be administered through any route commonly used in practice, e.g., orally, subcutaneously, sublingually, parenterally, per rectum, per and nares. The BH4 may be administered daily or at some other interval, e.g., every alternative day or even weekly. The BH4 is preferably administered in combination with a protein-restricted diet, and optionally concurrently with folates, including folate precursors, folic acids, and folate derivatives.

It is contemplated that that BH4 will be administered as part of a component of a therapeutic protein formulation. The protein-restricted diet may comprise a normal diet of low-protein containing foodstuff. Alternatively, the protein-restricted diet comprises an intake of protein formula that is phenylalanine-free protein diet, and the subject obtains his essential amount of Phe from remaining components of a very low protein diet. In certain embodiments, the protein-restricted diet is supplemented with non-phenylalanine containing protein supplements. More particularly, the non-phenylalanine containing protein supplements comprise tyrosine or other essential amino acids. In other embodiments, the protein supplements may also comprise folates, including folate precursors, folic acids, and folate derivatives.

The invention contemplates methods of treating an infant having phenylketonuria, comprising administering a stabilized BH4 composition to the patient in an amount effective to produce a decrease in the plasma phenylalanine concentration of the infant wherein the infant is between 0 and 3 years of age and the infant has a plasma phenylalanine concentration of between about 360  $\mu$ M to about 4800  $\mu$ M. Prior to the administering of BH4, the infant has a phenylalanine concen-

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tration of about 1200  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 1000  $\mu$ M. In other embodiments, prior to the administering of BH4 the infant has a phenylalanine concentration of about 800  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 600  $\mu$ M. In still further embodiments, prior to the administering of BH4 the infant has a phenylalanine concentration of about 400  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 300  $\mu$ M. The therapeutic methods contemplated herein should preferably reduce the plasma phenylalanine concentration of the infant to  $360 \pm 15$   $\mu$ M.

Also contemplated is a composition comprising a stabilized, crystalline form of BH4 that is stable at room temperature for more than 8 hours and a pharmaceutically acceptable carrier, diluent or excipient. The composition may further comprise a medical protein supplement. In other embodiments, the BH4 composition is part of an infant formula. In still other embodiments, the protein supplement is phenylalanine free. The protein supplement preferably is fortified with L-tyrosine, L-glutamine, L-carnitine at a concentration of 20 mg/100 g supplement, L-taurine at a concentration of 40 mg/100 g supplement and selenium. It may further comprise the recommended daily doses of minerals, e.g., calcium, phosphorus and magnesium. The supplement further may comprise the recommended daily dose of one or more amino acids selected from the group consisting of L-leucine, L-proline, L-lysine acetate, L-valine, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-tryptophan, L-serine, L-threonine, L-histidine, L-methionine, L-glutamic acid, and L-aspartic acid. In addition, the supplement may be fortified with the recommended daily dosage of vitamins A, D and E. The supplement preferably comprises a fat content that provides at least 40% of the energy of the supplement. Such a supplement may be provided in the form of a powder supplement or in the form of a protein bar.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

FIG. 1 is a powder X-ray diffraction pattern of (6R)-BH4 Form B.

FIG. 2 is a powder X-ray diffraction pattern of (6R)-BH4 Form A.

FIG. 3 is a powder X-ray diffraction pattern of (6R)-BH4 Form F.

FIG. 4 is a powder X-ray diffraction pattern of (6R)-BH4 Form J.

FIG. 5 is a powder X-ray diffraction pattern of (6R)-BH4 Form K.

FIG. 6 is a powder X-ray diffraction pattern of (6R)-BH4 Form C.

FIG. 7 is a powder X-ray diffraction pattern of (6R)-BH4 Form D.

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FIG. 8 is a powder X-ray diffraction pattern of (6R)-BH4 Form E.

FIG. 9 is a powder X-ray diffraction pattern of (6R)-BH4 Form H.

FIG. 10 is a powder X-ray diffraction pattern of (6R)-BH4 Form O.

FIG. 11 is a powder X-ray diffraction pattern of (6R)-BH4 Form G.

FIG. 12 is a powder X-ray diffraction pattern of (6R)-BH4 Form I.

FIG. 13 is a powder X-ray diffraction pattern of (6R)-BH4 Form L.

FIG. 14 is a powder X-ray diffraction pattern of (6R)-BH4 Form M.

FIG. 15 is a powder X-ray diffraction pattern of (6R)-BH4 Form N.

FIG. 16 is a mean blood phenylalanine level comparison at time zero, 3 days, and 7 days for multiple daily BH4 doses of 10 mg/kg/d and 20 mg/kg/d.

FIG. 17 is a comparison of daily individual blood phenylalanine levels for 12 adults having PKU and taking 10 mg/kg/d over 7 days.

FIG. 18 is a comparison of daily individual blood phenylalanine levels for 12 adults having PKU and taking 20 mg/kg/d over 7 days.

FIG. 19 is a comparison of daily individual blood phenylalanine levels for 8 children having PKU and taking 10 mg/kg/d over 7 days.

FIG. 20 is a comparison of daily individual blood phenylalanine levels for 8 children having PKU and taking 20 mg/kg/d over 7 days.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Dietary intervention is the therapeutic intervention used for all patients with severe classical PKU and in many patients with moderate PKU. However, such dietary protein restriction leads to an inadequate supply of protein, energy, vitamins and minerals to support normal growth and development. Thus, dietary protein restriction is at best an ineffective way of controlling the PKU in many classes of patients, especially in pregnant women and in young children, both categories of subjects that require elevated amounts of protein as compared to normal adult individuals. Use of dietary restriction also is hampered by poor patient compliance with a protein-restricted diet. In October 2000, the National Institutes of Health issued a consensus statement on PKU screening and management in which "research on nondietary alternatives to treatment of PKU [was] strongly encouraged." Thus, there is an art-recognized need for a therapeutic medicament to replace and/or supplement and alleviate the dietary restrictions under which a PKU patient is placed.

The present application for the first time describes a pharmaceutical intervention of PKU based on the administration of a stabilized form of BH4. The methods and compositions for producing such a stabilized BH4 compositions are described in further detail in Example 2. The stabilized BH4 compositions of the present invention comprise BH4 crystals that are stable at room temperature for longer than 8 hours. The methods and compositions of the present invention contemplate pharmaceutical compositions of the stabilized BH4 alone that may be delivered through any conventional route of administration, including but not limited to oral, intramuscular injection, subcutaneous injection, intravenous injection and the like. The compositions of the present invention may further comprise BH4 compositions in combination with an



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antioxidant that aids in prolonging the stability of the BH4 composition. In addition, discussed in greater below, the present invention further comprises foodstuffs that comprise BH4. For example, the invention contemplates conventional protein powder compositions such as PHENEX, LOFENALAC, PHENYL-FREE and the like that have been modified by the addition of BH4.

The present invention further contemplates the therapeutic intervention of various PKU phenotypes by administration of BH4 in combination with a protein-restricted diet. The BH4 to be administered in combination with the diet may, but need not necessarily, be a stabilized BH4 composition described herein. Those of skill in the art are aware of methods of producing a BH4 composition that is unstable at room temperature and in light. While therapies using such a composition are hindered by the instability of the BH4 composition, its use is still contemplated in certain combination therapies where BH4 non-responsive patients suffering from severe classical PKU are treated with a course of BH4 treatment and dietary protein restriction.

Methods and compositions for effecting the treatment of metabolic disorders, including PKU, are described in further detail herein below.

#### I. Patients to be Treated

The present invention is directed to the treatment of a variety of HPA patient populations with methods that comprise the use of stabilized BH4 compositions, or unstabilized BH4 compositions, either alone or in combination with other therapeutic regimens, for managing HPA and/or PKU. In particular, it is contemplated that any type of BH4, in a stabilized or other form may be used to treat that patient population that has phenylalanine concentrations that are low enough that dietary intervention is not normally used (i.e., patients with mild HPA). Such patients that are amenable to all forms treatment with BH4 compositions to ameliorate the effects of mild HPA, include pregnant women and infants with serum concentrations of less than 200  $\mu$ M. The various patient populations, and their different therapeutic needs, are discussed in further detail in the present section.

Certain embodiments of the present invention are directed to treating classic severe PKU by administering to the subject a protein-restricted diet in combination with a composition comprising BH4 or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of said subject as compared to said concentration in the absence of said combined administration. In addition, the invention also contemplates treating a pregnant female that has HPA by administering to the female a protein-restricted diet in combination with BH4 or a precursor or derivative thereof, such that the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the pregnant woman as compared to such a concentration in the absence of said combined administration. In specific embodiments, therapy is contemplated for patient who manifest Phe levels greater than 420  $\mu$ M

Other embodiments of the invention entail administering a stabilized BH4 composition to any individual that has HPA, characterized by a plasma Phe concentration greater than 180  $\mu$ M prior to the administration of the BH4, in an amount effective to produce a decrease in such a plasma Phe concentration of the patient. The methods of the invention also may be used in the treatment of PKU patients that have been diagnosed as unresponsive to a BH4 loading test. The methods of the invention will be useful in treating an infant having

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PKU characterized by an elevated Phe concentrations of between greater than 300  $\mu$ M/L with the stabilized BH4 compositions described herein. By "infant" the present application refers to a patient that is between the ages of 0 to about 36 months.

The data described herein demonstrates that subjects who are considered "unresponsive" to the single dose BH4 loading test may in fact respond to multiple doses of BH4 with a significant reduction in phenylalanine levels. Thus, another aspect of the invention provides a multiple dose loading test that involves administration of more than one dose of BH4. Exemplary multiple dose loading tests include administration of between 5 and 40 mg/kg tetrahydrobiopterin, or more preferably 10 to 20 mg/kg, over a time period of at least 1 day, or at least 2 days, or at least 3, 4, 5, 6, 7, 10 or 14 days, preferably 2-14, 3-14, or 5-10 days, and most preferably 7 days.

The invention provides methods of using any of the tetrahydrobiopterin polymorphs described herein, or stable pharmaceutical preparations comprising any of such polymorphs, for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described elsewhere herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal.

The invention further provides methods of using any of the polymorphs described herein, or stable pharmaceutical preparations comprising any of such polymorphs, for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from treatment with folates, including folate precursors, folic acids, or folate derivatives. Thus, it is also contemplated, that compositions and methods disclosed herein include the concurrent treatment with folates, including folate precursors, folic acids, or folate derivatives. Exemplary folates are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and salts thereof as is treatment with a pharmaceu-

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tical composition or foodstuff that comprises both a tetrahydrobiopterin polymorph and a folate.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (narrowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, such as hypertension, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, peripheral circulation disorder, coronary arteriole stenosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arteriosclerotic heart diseases or the like so far as they are associated with insulin resistance. It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

A. Characteristics of Severe Classical PKU and Methods of Treatment thereof According to the Present Invention.

As indicated herein above in the background section, severe PKU manifests in a plasma Phe concentration greater than 1200  $\mu\text{M/L}$  and may be found to be as high as 4800  $\mu\text{M/L}$ . Patients that have this disorder must be treated with a Phe-free diet in order to bring their plasma Phe concentrations down to a level that is clinically acceptable (typically, less than 600  $\mu\text{M/L}$ , and preferably less than 300  $\mu\text{M/L}$ ). These patients are only able to tolerate a maximum of between 250-350 mg dietary Phe per day (Spaapen et al., *Mol. Genet and Metab.* 78:93-99, 2003). As such, these patients are started on a Phe-restricted formula diet between 7-10 days after birth and are burdened with this dietary restriction for the remainder their lifespan. Any alleviation of the strict dietary restrictions that these individuals are encumbered with would be beneficial.

The tests used for the diagnosis of individuals with classical Phe are described in further detail below in Section III. These tests have revealed that patients with classical severe PKU are non-responsive to BH4 and require a low phenylala-

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anine diet (Lucke et al., *Pediatr. Neurol.* 28:228-230, 2003). In the present invention however, it is contemplated that this class of PKU patients should be treated with BH4 in order that the need for a strict phenylalanine-free diet may be alleviated.

Thus, it is contemplated that the methods of the invention will entail determining that the patient is suffering from classical PKU by monitoring the plasma Phe concentration of the individual. The patient is then treated by administering a combined regimen of a low protein diet and BH4 such that there is produced at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe concentration. Even more preferably, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with severe classical PKU has a Phe concentration of 4800  $\mu\text{M/L}$ , a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 480  $\mu\text{M/L}$ , a concentration that is sufficiently low to require little dietary restriction). Of course, it should be understood that the treatment methods of the present invention (whether for treating severe classical PKU or any other HPA described herein), should attempt to lower the plasma Phe concentrations of the patient to levels as close to 360  $\mu\text{M/L} \pm 15 \mu\text{M/L}$  as possible.

In preferred embodiments the plasma Phe concentrations of the classical PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is greater than 1000  $\mu\text{M/L}$  to any plasma Phe level that is less than 600  $\mu\text{M/L}$ . Of course, even if the combined treatment with the BH4 and the protein-restricted diet produces a lesser decrease in plasma Phe concentration, e.g., to a level of between 800  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$ , this will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-restricted formula, thereby resulting in a marked improvement in the quality of life of the individual, as well as leading to greater patient compliance with the dietary restriction.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy, the patient will be able to increase his/her intake of dietary Phe from 250-350 mg/day to 350-400 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a classic PKU patient to a moderate PKU patient). Of course, it would be preferable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 250-350 mg/day to 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a classic PKU patient to a mild PKU patient), or even more preferably, to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

B. Characteristics of BH4-Non Responsive PKU Patients and Methods of Treatment thereof According to the Present Invention.

A second group of patients that can be treated with the methods of the present invention are those individuals that have been determined to have an elevated plasma Phe concentrations i.e., any concentration that is greater than 200  $\mu\text{M/L}$ , but have been diagnosed to be non-responsive to BH4 therapy (as determined by the BH4 loading test described below). Such patients may include those individuals that have mild PKU (i.e., plasma Phe concentrations of up to 600  $\mu\text{M/L}$ ), individuals that have moderate PKU (i.e., plasma Phe

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concentrations of between 600  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$ ), as well as patients that have classic severe PKU (i.e., plasma Phe concentrations that are greater than 1200  $\mu\text{M/L}$ ).

The patients that are non-responsive to BH4 therapy are given BH4 in combination with a reduced amount of protein in their diet in order to decrease the plasma Phe concentrations of the patient. The methods of the present invention are such that the administration of the BH4 therapy produces a greater decrease in the plasma Phe concentrations of the patient as compared to the decrease that is produced with the same dietary protocol administered in the absence of the BH4 therapy.

In preferred embodiments, the patients are administered a composition that comprises a stabilized, crystallized form of BH4 characterized in Example 2 described herein below. This BH4 composition differs from those previously available in the art in that it is more stable at room temperature than the preparations previously known to those of skill in the art, e.g., those available in the BH4 loading kits (Schircks Laboratories, Jona, Switzerland.) Thus, the BH4 formulation may be stored at either room temperature or refrigerated and retain greater potency than the previously available BH4 compositions. As such, it is contemplated that this form of BH4 will have a greater therapeutic efficacy than a similar concentration the previously available BH4 compositions. This greater efficacy may be used to produce a therapeutically effective outcome even in patients that were previously identified as being non-responsive to BH4.

As with the subset of patients described in Section IA above, the BH4 non-responsive patients described in the present section may be treated by the stabilized BH4 compositions either alone or in combination with dietary restrictions. The dietary restrictions may be as a diet that restricts the Phe intake by providing a synthetic medical protein formula that has a diminished amount of Phe or alternatively, the dietary restriction may be one which simply requires that the patient limit his/her overall protein intake but nevertheless allows the patient to eat normal foodstuffs in limited quantities.

The preferred therapeutic outcomes discussed for classical PKU patients in Section IA above are incorporated into the present section by reference. Preferred therapeutic outcomes for patients with moderate PKU (i.e., patients that has an unrestricted plasma Phe concentration of 600  $\mu\text{M/L}$  to 1200  $\mu\text{M/L}$ ) include at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe concentration. Even more preferably, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with moderate classical PKU has a Phe concentration of 1000  $\mu\text{M/L}$ , a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 100  $\mu\text{M/L}$ , a concentration that is sufficiently low to require little dietary restriction).

In preferred embodiments, the plasma Phe concentrations of the moderate PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is between 600  $\mu\text{M/L}$  to 1200  $\mu\text{M/L}$  to any plasma Phe level that is less than 300  $\mu\text{M/L}$ . A particularly preferred treatment with the BH4 (either alone or in combination with a dietary restriction) produces a decrease in plasma Phe concentration, e.g., to a level of between 200  $\mu\text{M/L}$  to about 400  $\mu\text{M/L}$ , which will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-

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restricted formula. Indeed, in many studies, it is taught that such patients may even eat a normal diet.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 350-400 mg/day to 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a moderate PKU patient to a mild PKU patient). Of course, it would be preferable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 350-400 mg/day to 400 to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

Even if the patient being treated is one who manifests only mild PKU, i.e., has a dietary allowance of 400-600 mg Phe intake/day) will benefit from the BH4-based therapies of the present invention because it is desirable to produce a normalized plasma Phe concentration that is as close to 360  $\mu\text{M/L} \pm 15 \mu\text{M/L}$  as possible. For such patients, a preferred therapeutic outcomes will include at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe concentration. Even more preferably, the method will produce a 40%, 50%, 60%, or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with mild PKU has a Phe concentration of 600  $\mu\text{M/L}$ , a 60% decrease in the Phe concentration will produce a plasma Phe concentration of 360  $\mu\text{M/L}$ , i.e., an acceptable, normal concentration of plasma Phe).

In preferred embodiments, the plasma Phe concentrations of the mild PKU patient being treated is reduced from any amount of non-restricted plasma Phe concentration that is between 400  $\mu\text{M/L}$  to 600  $\mu\text{M/L}$  to any plasma Phe level that is less than 100  $\mu\text{M/L}$ . Of course, even if the treatment with the BH4 (either alone or in combination with a dietary restriction) produces a lesser decrease in plasma Phe concentration, e.g., to a level of between 200  $\mu\text{M/L}$  to about 400  $\mu\text{M/L}$ , this will be viewed as a clinically useful outcome of the therapy.

Any increase the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a mild PKU patient to a mild HPA patient) to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

Furthermore, even if the patient is one who only manifests the symptoms of non PKU HPA, i.e., has an elevated plasma Phe concentration of up to 600  $\mu\text{M/L}$ , but is otherwise allowed to eat a normal protein diet will benefit from the BH4 therapies of the invention because it has been shown that elevated Phe concentrations have significant effects on the IQ of such individuals. Moreover, as discussed below, BH4-based therapeutic intervention of subjects with special needs, e.g., pregnant women and infants, is particularly important even if that patient's plasma Phe levels are within the perceived "safe" level of less than 200  $\mu\text{M/L}$ .

C. Maternal PKU and Methods of Treatment thereof According to the Present Invention.

Metabolic control of plasma Phe levels in PKU women planning conception and those who are pregnant is important



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because of the serious consequences to the fetus exposed to even moderately elevated Phe levels in utero, regardless of the PAH status of the fetus. Therapeutic control of plasma Phe concentration is especially important in the first trimester of pregnancy, as failure to achieve adequate control will result in disorders including microcephaly, mental deficiency and congenital heart disease.

For example, the NIH Consensus Statement (vol 17 #3, October 2000) on Phenylketonuria reported that exposure of a fetus to maternal Phe levels of 3-10 mg/dL produced a 24% incidence of microcephaly, whilst those exposed to greater than 20 mg/dL (i.e., greater than 1200  $\mu$ M/L) had a 73% incidence of microcephaly. Likewise congenital heart disease was found in over 10% of children exposed to maternal Phe levels that were greater than 20 mg/dL. Importantly, it has been noted that levels of Phe greater than 6 mg/dL significantly decrease the IQ of the child. Thus, it is imperative to ensure that the plasma Phe concentration of women with all forms of phenylketonuria, even those manifesting the mildest HPA, must be tightly controlled in order to avoid the risk of maternal PKU syndrome. However, the acceptable target levels for the plasma Phe concentrations of PKU women that have been used in U.S. clinics have ranged between 10 mg/dL and 15 mg/dL, which are much higher than the 2-6 mg/dL levels recommended for pregnant women or the 1-4 mg/dL that are used in British and German clinics to diminish the risks of developing maternal PKU syndrome.

Another important consideration for pregnant women is their overall protein intake. During pregnancy, it is important that women eat sufficient protein because it has been suggested that a low protein diet during pregnancy will result in retarded renal development and subsequent reduction in the number of nephrons and potentially leads to hypertension in adulthood. (D'Agostino, *N. Engl. J. Med.* 348(17):1723-1724, 2003). The following table provides exemplary guidelines for the recommended total dietary protein intake for various individuals.

TABLE

United States Guidelines for dietary protein requirements		
	Age	Recommended Total Protein Intake (g)
Infant	6 months or less	13
	6 months-1 year	14
Children	1-3 years	16
	4-6 years	24
	7-10 years	28
Males	11-14 years	45
	15-18 years	59
	19-24	58
	25-50	63
	51+	63
Females	11-14 years	46
	15-18 years	44
	19-24	46
	25-50	50
	51+	50
Pregnant		60
Lactating		65

The actual amount of protein ingested depends on the Phe content of the protein. The amino acid profiles of plant proteins is different from animal proteins. For example, with a focus on starches and vegetables, a general rule of 45-50 mg/Phe per gram of protein may suffice. However, an accepted standard for evaluating the constituents amino acids of a protein is an egg white, which contains 3.5 grams of protein of which 204 mg is Phe.

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As can be seen from the above exemplary guidelines, in the United States, the recommended protein intake for women of child-bearing age (e.g., less than 51) is from about 44 to 50 g/day, whereas pregnant women require are recommended an intake of about 60 g/day. In Canada and the United Kingdom, the recommended protein intake for pregnant women is in the order of about 70 g/day and 52 g/day. Thus, the need to ensure that the plasma Phe concentration levels of pregnant women are tightly controlled is further complicated by the fact that this group of PKU patient requires more protein than non-pregnant PKU females of comparable age.

In view of the above, it is contemplated that BH4-based therapies of the present invention will be particularly useful in pregnant women. It is contemplated that a woman suffering from any form of HPA who is pregnant or is contemplating pregnancy will be placed on a course of BH4 therapy to ensure that her plasma Phe concentration levels are maintained as close to 180  $\mu$ M/L to about 360  $\mu$ M/L as possible. Such a course of therapy will preferably allow that woman to increase her level of normal protein intake.

The discussion of levels of plasma Phe concentrations and the degrees to which such Phe concentrations should be decreased discussed herein above in Sections IA and IB are incorporated into the present section for pregnant women.

D. Managing PKU in Infants and Methods of Treatment thereof According to the Present Invention.

As discussed herein throughout, it has been determined that an elevation in the plasma Phe concentration in infants (ages zero to 3 years old) results in significant drop in IQ of the child. However, as has been discussed elsewhere in the specification, patients that have an elevated plasma Phe concentration of anywhere up to 400  $\mu$ M/L do not normally receive any dietary intervention. Thus, infants at the age of zero to 3 years in age suffer from significant deleterious effects from the present therapies. The instant application contemplates treating any infant having an unrestricted plasma Phe concentration that is greater than 360  $\mu$ M/L  $\pm$  15  $\mu$ M/L with a therapeutic composition that comprises BH4 in order to produce a beneficial decrease the plasma Phe concentration of that subject.

In preferred embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of about 1200  $\mu$ M/L prior to the administration of BH4 and said administration decreases the plasma Phe concentration. Preferably, the plasma Phe concentration is decreased to from greater than 1800 to about 1500  $\mu$ M/L, about 1200  $\mu$ M/L, about 1100  $\mu$ M/L, about 1000  $\mu$ M/L, about 900  $\mu$ M/L, about 800  $\mu$ M/L, about 700  $\mu$ M/L, about 600  $\mu$ M/L, about 550  $\mu$ M/L, about 500  $\mu$ M/L, about 450  $\mu$ M/L, 400  $\mu$ M/L, about 350  $\mu$ M/L, about 300  $\mu$ M/L, about 275  $\mu$ M/L, about 250  $\mu$ M/L upon administration. In other embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of greater than 1200  $\mu$ M/L and preferably, this plasma Phe concentration is decreased to about 800  $\mu$ M/L, or more preferably to about 500  $\mu$ M/L or even more preferably to about 360  $\mu$ M/L upon administration of BH4, either alone or in combination with diet. Those of skill in the art would understand that the invention contemplates treating infants with unrestricted plasma Phe concentrations of greater than 360  $\mu$ M/L with BH4 to produce decreases in such plasma Phe concentrations. The discussion of therapeutic reductions of plasma Phe concentrations in Sections IA and IB above are incorporated herein by reference. Further, any decrease over 10% of the initial unrestricted plasma Phe concentration will be considered a therapeutic outcome for the therapeutic regimens for the infants. It should be understood that the BH4 therapies may

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be combined with dietary restrictions to effect the therapeutic decrease in plasma Phe concentrations in such infants.

## II. Compositions for Use in the Treatment

The present invention contemplates therapeutic intervention of PKU/HPA. Such intervention is based initially on the use of BH4. The BH4 may be used alone or in combination with dietary restrictions. Further the BH4 and/or dietary restrictions may further be combined with other therapeutic compositions that are designed, for example to combat other manifestations of PKU, such as for example, large neutral amino acids to prevent Phe accumulation in the brain (see Koch et al., *Mol. Genet. Metabol.* 79:110-113, 2003) or tyrosine supplementation. The present section provides a discussion of the compositions that may be used in the treatments contemplated herein.

### A. BH4 Compositions

BH4 is a cofactor in Phe hydroxylation and prior to the present invention, it was shown that less than 2% of patients having an elevated Phe at birth have defects in BH4 synthesis. With those individuals that were identified as being BH4 responsive, it was suggested that the patients would be non-responsive to dietary intervention and hence, those individuals were fed a normal diet but given BH4 therapy alone. Thus, prior to the present invention, there was much skepticism in the art as to the therapeutic benefits of BH4 administration to PKU/HPA patients. However, as discussed herein throughout, BH4 may be administered for a therapeutic intervention of patients that have been diagnosed as non-BH4 responsive. Moreover, the present inventors show that BH4 therapy can be combined with dietary restrictions to produce a therapeutic outcome in both individuals that are responsive to a BH4 loading test as well as individuals that are non-responsive to BH4 loading.

U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408 and Canadian application CA 2420374 (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivative thereof that may be used as compositions for the present invention. Any such methods may be used to produce BH4 compositions for use in the therapeutic methods of the present invention.

U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment of PKU/HPA as described herein.

In addition to the above general methods of making BH4, the present invention particularly contemplates making and using a BH4 composition which is a stabilized BH4 composition. Preferably the stabilized BH4 composition is in crystalline form. Methods of making the stabilized BH4 compositions for use in the present invention are described in Example 2. Such a crystalline form may prove useful as an additive to conventional protein formulas for the treatment of PKU. The crystalline form also may conveniently be formed into a tablets, powder or other solid for oral administration.

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The forms and routes of administration of BH4 are discussed in further detail in the Pharmaceutical Compositions section below.

In preferred embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

Regardless of the amount of BH4 administered, it is desirable that the administration decreases the plasma Phe concentration of the patients to the concentrations discussed in Section I for the various types of patients.

### B. Dietary Protein

In addition to administering BH4 and related analogs to HPA/PKU patients, it is contemplated that the dietary protein of the patients also may be restricted or modified. Those of skill in the art are aware of various commercially available protein formulas for use in the treatment of PKU. Such formulas include MAXIMAID, PHENEX 1, PHENEX 2 (Ross Laboratories, Liverpool, UK), LOFENALAC, PHENYL-FREE (Mead-Johnson), and the like.

Those of skill in the art may use the referenced protein formulas, which are generally free of Phe concentrations. The protein formulas often are supplemented with amino acids that are deficient in PKU patients. Such amino acids include, for example, L-tyrosine, and L-glutamine. It has been suggested that it may be desirable to supplement the diet of PKU patients with valine, isoleucine and leucine (see U.S. Pat. No. 4,252,822). In certain clinical manifestations, the toxic effects of PKU are caused by Phe blocking the brain uptake of other amino acids such as tyrosine and tryptophan. It has been found that supplementing the diet of a PKU patient with excess of such large neutral amino acids blocks Phe uptake into the brain and lowers brain Phe levels. Thus, it is contemplated that for the methods of the present invention, the dietary regimen may further be supplemented with compositions that comprise one or more of these amino acids (Koch et al., *Mol. Genet. Metabol.* 79:110-113, 2003).

Further, as it is known that L-carnitine and taurine which are normally found in human milk and other foodstuffs of animal origin also should be supplied in addition to the protein restriction. In certain embodiments, the L-carnitine may be supplied as 20 mg/100 g of protein supplement, and the taurine may be supplied as 40 mg/100 g protein supplement in order to help supply amounts of these factors normally found in human milk and foods of animal origin.

In addition, those of skill in the art are by reference to the 2000 National Academy of Sciences-National Research Council Dietary Reference Intakes for a further listing of other components, such as essential vitamins and minerals that should be supplied to the patient to ensure that other supplements are being provided despite the dietary protein restriction.

Referring to the Table presented in Section IC above for total protein amounts and the figures presented in Section I in general for the desirable plasma Phe concentrations, one of skill in the art will be able to determine the amount of dietary

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protein restriction that is required and thus adjust the diet of the patient accordingly. Taking for example, a male of about 11-14 years of age, that individual should preferably receive 45 g protein/day. In the event that the individual is one that has severe classic PKU, his unrestricted plasma Phe concentration will likely be greater than 1200  $\mu\text{M/L}$ , and most, if not all of the dietary protein source for that individual is likely to be from a powdered protein supplement, which preferably lowers his plasma Phe concentrations to less than 600  $\mu\text{M/L}$ . By administering BH4 to that subject, a therapeutic outcome would be one which produces greater decrease in the plasma Phe concentrations of patient or alternatively, the therapeutic outcome is one in which the individual's plasma Phe concentrations is lowered to a similar degree, but that individual is able to tolerate protein from a normal diet rather than from a dietary formula.

Similarly, a male of about 11-14 years of age, is one who has moderate PKU, it may be possible using the methods of the present invention to give him the allotted 45 g protein/day through a normal protein intake rather than a restricted formula. Determining whether the methods of the invention are effective will entail determining the plasma Phe concentrations of the patient on a regular basis to ensure that the plasma Phe concentrations remain below at least 400  $\mu\text{M/L}$ . Tests for determining such concentrations are described below. Preferably, concentrations of less than or about 360  $\mu\text{M/L}$  are achieved.

### III. Identifying and Monitoring Patient Populations

As discussed herein throughout, it will be necessary in various embodiments of the present invention to determine whether a given patient is responsive to BH4 therapy, and to determine the phenylalanine concentrations of the patient both initially to identify the class of PKU patient being treated and during an ongoing therapeutic regimen to monitor the efficacy of the regimen. Exemplary such methods are described herein below.

#### A. BH4 Loading Test

In order to identify a patient as being responsive to BH4, those of skill in the art perform a "BH4 loading" test. Two types of loading tests have been used to achieve the differential diagnosis of HPA. The first is a simple oral BH4 loading test and the second is a combined phenylalanine/BH4 loading test.

The simplest BH4 loading test is one in which exogenous BH4 is administered and the effects of the administration on lowering of plasma Phe concentrations is determined. Intravenous loading of 2 mg/kg BH4 was initially proposed by Danks et al., (*Lancet* 1:1236, 1976), as BH4 of greater purity has become available it has become possible to perform the test using an oral administration of BH4 in amounts of about 2.5 mg/kg body weight. Ultimately, a standardized approach was proposed by Niederwieser et al. in which a 7.5 mg/kg single oral dose of BH4 is administered (*Eur. J. Pediatr.* 138:441, 1982), although some laboratories do still use upwards of 20 mg BH4/kg body weight. This test allows discrimination between patients that have HPA due to a deficit in BH4 or through a deficiency in PAH.

In order for the simple BH4 loading test to produce reliable results, the blood Phe levels of the patient need to be higher than 400  $\mu\text{M/L}$ . Therefore, it is often customary for the patient to be removed from the PKU diet for 2 days prior to performing the loading test. A BH4 test kit is available and distributed by Dr. Schircks Laboratories (Jona, Switzerland). This kit recommends a dosage of 20 mg BH4/kg body weight about 30 minutes after intake of a normal meal.

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As indicated above, the Phe concentration of a patient ideally needs to be higher than 400  $\mu\text{M/L}$  in order to obtain an accurate BH4 reading. In the combined Phenylalanine/BH4 loading test, an oral administration of Phe (100 mg/kg body weight) plus BH4 (20 mg/kg body weight) allows selective screening of all BH4 deficiencies. Typically, the Phe is administered in an oral dose and it is followed approximately one hour later with BH4. The plasma Phe levels are monitored before and at convenient time intervals (e.g., 1, 3, 5, 9, 13 and 25 hours) post-Phe administration.

In either the simple BH4 loading test or the combined Phe/BH4 loading test, it has been suggested that a decrease in plasma Phe of more than 30% of the plasma Phe value prior to BH4 challenge within 24 hours post-load is indicative of BH4 responsiveness (Spaapen et al, *Mol. Genet. and Metabol.*, 78:93-99, 2003).

Other methods of performing BH4 loading tests also may be used. Exemplary such tests are described in e.g., Muntau et al., (*N. Engl. J. Med.* 347(26):2002) and Bernegger and Blau (*Mol. Genet. Metabol.* 77:304-313, 2002).

In Bernegger and Blau, the BH4 loading test uses 20 mg/kg BH4 and blood sampling for phenylalanine and tyrosine is performed at 0, 4, 8, and 24 hours to differentiate between BH4-responders and non-responders. The test is carried out after at least 3 hours of fasting. Urine samples of neopterin and biopterin are tested before the test. After an oral application of 6R BH4 (20 mg/kg body weight), normal food intake is allowed during the entire testing period. Blood samples are assayed for Phe and Tyr measurements at 0, 4, 8 and 24 hours. Another urine sample is collected between 4-8 hours. Dihydropteridine reductase activity also may be measured anytime during the test. In patients that have plasma phenylalanine levels less than 400  $\mu\text{M/L}$  or patients already on a low-phenylalanine diet, Bernegger and Blau recommend a combined phenylalanine-BH4 test in which 100 mg Phe/kg body weight is administered orally 3 hours before the BH4 administration.

Bernegger and Blau calculated BH4-responsiveness as "phenylalanine hydroxylation" at 4 and 8 hours after loading and was expressed as a percentage of the phenylalanine eliminated. The slope (S) of the graphs of "hydroxylation rates" at 0, 4 and 8 hours are compared for different BH4 products and different groups of patient. The slope discriminates between non-responders, slow responders and responders. The slow responders (see FIG. 5 in Bernegger and Blau) need more time to reach the cut-off values of 360  $\mu\text{M/L}$  and that the effectiveness of administered BH4 depends on the initial phenylalanine levels. These authors recommend that for some patients with plasma Phe of less than 800  $\mu\text{M/L}$  and for most patients with a plasma Phe greater than 1200  $\mu\text{M/L}$ , a Phe measurement should be taken at 21 hours. A plot of Phe/S vs. time can be used to estimate the time needed to reach the therapeutic "safe" plasma Phe values of less than 360  $\mu\text{M/L}$ .

Muntau et al. (2002) also provide exemplary BH4 loading tests that can be used to calculate the times and concentrations of BH4 administration. Again these authors employed a combined PHE/BH4 loading test in which patients are given a meal that contains 100 mg Phe/kg body weight. One hour after the meal, the patients are given an oral dose of 20 mg/kg BH4 (Schircks Laboratories). Blood phenylalanine concentrations are determined by electrospray ionization mass spectrometry before Phe loading as well as before, and at 4, 8, and 15 hours after BH4 loading. Newborns may be breast fed, whereas older patients are given a standardized protein intake (10 mg Phe/kg) between 6-8 hours after BH4 loading. Muntau also describe methods for Phe oxidation. After a 4-hour fast and an overnight fast a total of 6 mg/kg 13C labeled Phe dissolved in dextrose solution is administered orally. Breath samples are



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then collected over a period of 180 minutes and stored in evacuated glass tubes. The samples are then analysed using isotope ratio mass spectrometry and the recovery of carbon 13 is calculated (Treacy et al., *Pediat. Res.* 42:430-5, 1997)

Muntau et al. classify patients as BH4 responsive when the blood Phe levels 15 hours post-BH4 challenge have decreased by more than 30% from the value obtained prior to the BH4 administration. An improvement in the rate of Phe oxidation, as determined by measurements of carbon dioxide obtained during the 180 minutes of testing, was considered significant when the supplementation with BH4 increased the value of Phe oxidation by at least 15%.

Those of skill in the art will be able to use any of the above-referenced methods to determine whether an individual will be responsiveness to BH4. However, other equivalent and related methods for determining BH4 responsiveness also may be known to those of skill in the art and may be used instead of the methods described above.

#### B. Determination of Phe Concentrations

There are numerous methods for determining the presence of Phe in blood (Shaw et al., *Analytical Methods in Phenylketonuria-Clinical Biochemistry*, In Bickett et al. Eds. *Phenylketonuria and Some Other Inborn Errors of Amino Acid Metabolism*, Stuttgart, Georg Thieme Verlag, 47-56 (1971)). Typically, phenylalanine and tyrosine concentrations are determined from the serum of a patient using a fluorometric assay. This assay relies on the formation of fluorescent substance when phenylalanine is heated with ninhydrin in the presence of leucylalanine (McCaman et al., *J. Lab. Clin. Med.* 59:885-890, 1962.)

The most popular method for determining Phe concentrations is the Guthrie test in which discs are punctured from filter paper that has been saturated with a blood sample from the patient. The uniform discs are incubated in a tray of agar that has been seeded with *Bacillus subtilis* and contains a specific inhibitor of *Bacillus subtilis* growth. As the phenylalanine transfers from the uniform discs onto the agar, the Phe reverse the inhibition of bacterial growth thereby yielding an area of bacterial growth that can be correlated to phenylalanine concentration by comparison to similar assays performed using discs containing known amounts of Phe.

Other methods of quantifying Phe concentration include HPLC, mass spectrometry, thin layer chromatography and the like. Such methods can be used to determine the plasma Phe concentration of a patient before the therapy and to monitor the Phe concentration during the therapeutic regimen to determine the efficacy thereof.

It is contemplated that the plasma Phe levels of the patients will be monitored at convenient intervals (e.g., daily, every other day or weekly) throughout the time course of the therapeutic regimen. By monitoring the plasma Phe levels with such regularity, the clinician will be able to assess the efficacy of the treatment and adjust the BH4 and/or dietary protein requirements accordingly.

#### IV. Combination Therapy

Certain methods of the invention involve the combined use of BH4 and dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. To achieve the appropriate therapeutic outcome in the combination therapies contemplated herein, one would generally administer to the subject the BH4 composition and the dietary restriction in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve

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administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4. The BH4 also may be formulated into a protein bar or other foodstuff such as brownies, pancakes, cake, suitable for ingestion.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, with a delay time of only about 1 hour being most preferred. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Gent. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4):243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

#### V. Pharmaceutical Compositions

Pharmaceutical compositions for administration according to the present invention can comprise a first composition comprising BH4 in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by any means that achieve their intended purposes. Amounts and regimens for the administration of a composition according to the present invention can be determined readily by those with ordinary skill in the art for treating PKU. As discussed above,

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those of skill in the art could initially employ amounts and regimens of BH4 currently being proposed in a medical context, e.g., those compositions that are being proposed for modulating NOS activity, or for use in the treatment of pain or depression as discussed in the patents listed in Section II

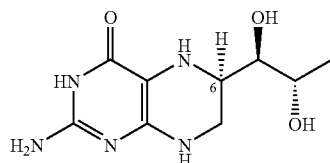
Any of the protocols, formulations, routes of administration and the like described that have been used for administering BH4 for loading tests can readily be modified for use in the present invention.

The compositions and methods described herein are not limited to the use of a particular form of BH4, or form of an analog or derivative of BH4. Indeed, it is contemplated that the compositions and methods within the scope of this invention include all compositions comprising any form BH4, and any form of an analog or derivative thereof in an amount effective to achieve its intended purpose. Nonlimiting examples of analogs for use in the compositions and methods described herein include pteridine, pterin, neopterin, bipterin, 7,8-Dihydrobiopterin, 6-methyltetrahydropterin, and other 6-substituted tetrahydropterin and other 6-substituted tetrahydropterins, sepiapterin, 6,7-Dimethyltetrahydropterin, 6-methyl bipterin and other 6-substituted bipterins, and other analogs that are described in the art. Nonlimiting examples of derivatives for use in the compositions and methods described herein include the derivatives described in U.S. Pat. Nos. 4,758,571; 4,774,244; 6,162,806; 5,902,810; 2,955,110; 2,541,717; 2,603,643; and 4,371,514, the disclosures of which are hereby incorporated herein.

Certain therapeutic methods of the present invention contemplate a combination therapy in which BH4-based compositions are administered in addition to a modified protein diet, the pharmaceutical compositions of the invention also contemplate all compositions comprising at least BH4-based therapeutic agent, analog or homologue thereof in an amount effective to achieve the amelioration of one or more of the symptoms of PKU when administered in combination with the modified protein diet. Of course, the most obvious symptom that may be alleviated is that the combined therapy produces a decrease in the plasma Phe concentration, however, other symptoms such as changes in IQ, executive function, concentration, mood, behavioral stability job performance and the like also may be monitored. Such indicia are monitored using techniques known to those of skill in the art.

#### Crystalline Polymorphs of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

It has been found that BH4, and in particular, the dihydrochloride salt of BH4, exhibits crystal polymorphism. The structure of BH4 is shown below:



The (6R) form of BH4 is the known biologically active form, however, BH4 is also known to be unstable at ambient temperatures. It has been found that one crystal polymorph of BH4 is more stable, and is stable to decomposition under ambient conditions.

BH4 is difficult to handle and it is therefore produced and offered as its dihydrochloride salt (Schircks Laboratories, Jona, Switzerland) in ampoules sealed under nitrogen to pre-

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vent degradation of the substance due to its hygroscopic nature and sensitivity to oxidation. U.S. Pat. No. 4,649,197 discloses that separation of (6R)- and 6(S)-L-erythro-tetrahydrobiopterin dihydrochloride into its diastereomers is difficult due to the poor crystallinity of 6(R,S)-L-erythro-tetrahydrobiopterin dihydrochloride. The European patent number 0 079 574 describes the preparation of tetrahydrobiopterin, wherein a solid tetrahydrobiopterin dihydrochloride is obtained as an intermediate. S. Matsuura et al. describes in Chemistry Letters 1984, pages 735-738 and Heterocycles, Vol. 23, No. 12, 1985 pages 3115-3120 6(R)-tetrahydrobiopterin dihydrochloride as a crystalline solid in form of colorless needles, which are characterized by X-ray analysis disclosed in J. Biochem. 98, 1341-1348 (1985). An optical rotation of 6.81° was found the crystalline product, which is quite similar to the optical rotation of 6.51° reported for a crystalline solid in form of white crystals in example 6 of EP-A2-0 191 335.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride indicated that the compound may exist in different crystalline forms, including polymorphic forms and solvates. The continued interest in this area requires an efficient and reliable method for the preparation of the individual crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and controlled crystallization conditions to provide crystal forms, that are preferably stable and easy to handle and to process in the manufacture and preparation of formulations, and that provide a high storage stability in substance form or as formulated product, or which provide less stable forms suitable as intermediates for controlled crystallization for the manufacture of stable forms.

#### Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B." Results obtained during investigation and development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride development revealed that there are several known crystalline solids have been prepared, but none have recognized the polymorphism and its effect on the stability of the BH4 crystals.

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application, which are prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

As used herein, the following abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity. A characteristic X-ray powder diffraction pattern is exhibited in FIG. 1.

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It has been found that other polymorphs of BH4 have a satisfactory chemical and physical stability for a safe handling during manufacture and formulation as well as providing a high storage stability in its pure form or in formulations. In addition, it has been found that form B, and other polymorphs of BH4 can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive crystal form B, can be used for the preparation of the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms than polymorph form B, such as polymorph A, in suitable polar and non aqueous solvents. Thus, the pharmaceutical preparations described herein refers to a preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Other forms of BH4 can be converted for form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methy-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallisation from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water: acetic acid: tetrahydrofuran of 1:3:2 to; 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallisation from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallisation and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is

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prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application.

#### Polymorph Form A

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form A," or "polymorph A." Polymorph A is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph A is a hygroscopic anhydrate which is a meta-stable form with respect to form B; however, it is stable over several months at ambient conditions if kept in a tightly sealed container. Form A is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form A can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Polymorph A which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) of: 15.5 (vs), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m), 3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w). FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph A exhibits a characteristic Raman spectra bands, expressed in wave numbers (cm<sup>-1</sup>) at: 2934 (w) 2880 (w), 1692 (s), 1683 (m), 1577 (w), 1462 (m), 1360 (w), 1237 (w), 1108 (w), 1005 (vw), 881 (vw), 813 (vw), 717 (m), 687 (m), 673 (m), 659 (m), 550 (w), 530 (w), 492 (m), 371 (m), 258 (w), 207 (w), 101 (s), 87 (s) cm<sup>-1</sup>.

Polymorph form A may be obtained by freeze drying or water removal of solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water. Polymorph form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at ambient temperatures in water, (1) cooling the solution to low temperatures for solidifying the solution, and removing water under reduced pressure, or (2) removing water from said aqueous solution.



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The crystalline form A can be isolated by filtration and then dried to evaporate absorbed water from the product. Drying conditions and methods are known and drying of the isolated product or water removal pursuant to variant (2) described herein may be carried out in applying elevated temperatures, for example up to 80° C., preferably in the range from 30° C. to 80° C., under vacuum or elevated temperatures and vacuum. Prior to isolation of a precipitate obtained in variant (2), the suspension may be stirred for a certain time for phase equilibration. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 5 to 40 percent by weight, by reference to the solution.

A fast cooling is preferred to obtain solid solutions as starting material. A reduced pressure is applied until the solvent is completely removed. Freeze drying is a technology well known in the art. The time to complete solvent removal is dependent on the applied vacuum, which may be from 0.01 to 1 mbar, the solvent used and the freezing temperature.

Polymorph form A is stable at room temperature or below room temperature under substantially water free conditions, which is demonstrated with phase equilibration tests of suspensions in tetrahydrofuran or tertiary-butyl methyl ether stirred for five days and 18 hours respectively under nitrogen at room temperature. Filtration and air drying at room temperature yields unchanged polymorph form A.

#### Polymorph Form F

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form F," or "polymorph F." Polymorph F is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph F is a meta-stable form and a hygroscopic anhydrate, which is more stable than form A at ambient lower temperatures and less stable than form B at higher temperatures and form F is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form F can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Polymorph F exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 17.1 (vs), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w), 3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 2.96 (m). FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph F may be obtained by phase equilibration of suspensions of polymorph form A in suitable polar and non-aqueous solvents, which scarcely dissolve said lower energy forms, especially alcohols such as methanol, ethanol, propanol and isopropanol. Polymorph form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can also be prepared by dispersing particles of solid form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-aqueous solvent that scarcely dissolves said (6R)-L-erythro-tetrahydrobiopterin dihydrochloride below room temperature, stirring the suspension at said temperatures for a time sufficient to produce polymorph form F, thereafter isolating crystalline form F and removing the solvent from the isolated form F. Removing of solvent and drying may be carried out under air, dry air or a dry protection gas such as nitrogen or noble gases and at

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or below room temperature, for example down to 0° C. The temperature during phase equilibration is preferably from 5 to 15° C. and most preferably about 10° C.

#### Polymorph Form J

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form J," or "polymorph J." The polymorph J is slightly hygroscopic and adsorbs water when handled at air humidity. The polymorph J is a meta-stable form and a hygroscopic anhydrate, and it can be transformed back into form E described below, from which it is obtained upon exposure to high relative humidity conditions such as above 75% relative humidity. Form J is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form J can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Form J exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs). FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph J may be obtained by dehydration of form E at moderate temperatures under vacuum. In particular, polymorph form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by taking form E and removing the water from form E by treating form E in a vacuum drier to obtain form J at moderate temperatures which may mean a temperature in the range of 25 to 70° C., and most preferably 30 to 50° C.

#### Polymorph Form K

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form K," or "polymorph K." Polymorph K is slightly hygroscopic and adsorbs water to a content of about 2.0 percent by weight, which is continuously released between 50° C. and 100° C., when heated at a rate of 10° C./minute. The polymorph K is a meta-stable form and a hygroscopic anhydrate, which is less stable than form B at higher temperatures and form K is especially suitable as intermediate and starting material to produce stable polymorph forms, in particular form B. Polymorph form K can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Form K exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m). FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph K may be obtained by crystallization from mixtures of polar solvents containing small amounts of water and in the presence of small amounts of ascorbic acid. Solvents for the solvent mixture may be selected from acetic acid and an alcohol such as methanol, ethanol, n- or isopropanol. In particular, polymorph form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a



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mixture of acetic acid and an alcohol or tetrahydrofuran containing small amounts of water and a small amount of ascorbic acid at elevated temperatures, lowering temperature below room temperature to crystallize said dihydrochloride, isolating the precipitate and drying the isolated precipitate at elevated temperature optionally under vacuum. Suitable alcohols are for example methanol, ethanol, propanol and isopropanol, whereby ethanol is preferred. The ratio of acetic acid to alcohol or tetrahydrofuran may be from 2:1 to 1:2 and preferably about 1:1. Dissolution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be carried out in presence of a higher water content and more of the anti-solvent mixture can be added to obtain complete precipitation. The amount of water in the final composition may be from 0.5 to 5 percent by weight and the amount of ascorbic acid may be from 0.01 to 0.5 percent by weight, both by reference to the solvent mixture. The temperature for dissolution may be in the range from 30 to 100 and preferably 35 to 70° C. and the drying temperature may be in the range from 30 to 50° C. The precipitate may be washed with an alcohol such as ethanol after isolation, e.g., filtration. The polymorph K can easily be converted in the most stable form B by phase equilibration in e.g. isopropanol and optionally seeding with form B crystals at above room temperature such as temperatures from 30 to 40° C.

#### Hydrate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline hydrate, which shall be described and defined herein as forms C, D, E, H, and O. These hydrate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

#### Hydrate Form C

It has been found that a hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form C," or "hydrate C." The hydrate form C is slightly hygroscopic and has a water content of approximately 5.5 percent by weight, which indicates that form C is a monohydrate. The hydrate C has a melting point near 94° C. ( $\Delta H_f$  is about 31 J/g) and hydrate form C is especially suitable as intermediate and starting material to produce stable polymorphic forms. Polymorph form C can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form C exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m). FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form C may be obtained by phase equilibration at ambient temperatures of a polymorph form such as polymorph B suspension in a non-solvent which contains water in an amount of preferably about 5 percent by weight, by reference to the solvent. Hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by suspending (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-solvent such as, heptane, C1-C4-alcohols such as

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methanol, ethanol, 1- or 2-propanol, acetates, such as ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or binary or ternary mixtures of such non-solvents, to which sufficient water is added to form a monohydrate, and stirring the suspension at or below ambient temperatures (e.g., 0 to 30° C.) for a time sufficient to form a monohydrate. Sufficient water may mean from 1 to 10 and preferably from 3 to 8 percent by weight of water, by reference to the amount of solvent. The solids may be filtered off and dried in air at about room temperature. The solid can absorb some water and therefore possess a higher water content than the theoretical value of 5.5 percent by weight. Hydrate form C is unstable with respect to forms D and B, and easily converted to polymorph form B at temperatures of about 40° C. in air and lower relative humidity. Form C can be transformed into the more stable hydrate D by suspension equilibration at room temperature.

#### Hydrate Form D

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form D," or "hydrate D." The hydrate form D is slightly hygroscopic and may have a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form D is a monohydrate. The hydrate D has a melting point near 153° C. ( $\Delta H_f$  is about 111 J/g) and is of much higher stability than form C and is even stable when exposed to air humidity at ambient temperature. Hydrate form D can therefore either be used to prepare formulations or as intermediate and starting material to produce stable polymorph forms. Polymorph form D can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form D exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w). FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form D may be obtained by adding at about room temperature concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents, and stirring the suspension at ambient temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding at about room temperature a concentrated aqueous solution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. Form D contains a small excess of water, related to the monohydrate, and it is believed that it is absorbed water due to the slightly hygroscopic nature of this crystalline hydrate. Hydrate form D is deemed to be the most stable one under the known hydrates at ambient temperatures and a

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relative humidity of less than 70%. Hydrate form D may be used for formulations prepared under conditions, where this hydrate is stable. Ambient temperature may mean 20 to 30° C.

#### Hydrate Form E

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form E,” or “hydrate E.” The hydrate form E has a water content of approximately 10 to 14 percent by weight, which suggests that form E is a dihydrate. The hydrate E is formed at temperatures below room temperature. Hydrate form E is especially suitable as intermediate and starting material to produce stable polymorph forms. It is especially suitable to produce the water-free form J upon drying under nitrogen or optionally under vacuum. Form E is non-hygroscopic and stable under rather high relative humidities, i.e., at relative humidities above about 60% and up to about 85%. Polymorph form E can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form E exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w). FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form E may be obtained by adding concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent cooled to temperatures from about 10 to -10° C. and preferably between 0 to 10° C. and stirring the suspension at said temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent which is cooled to temperatures from about 10 to -10° C., and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. A preferred non-solvent is tetrahydrofuran. Another preparation process comprises exposing polymorph form B to an air atmosphere with a relative humidity of 70 to 90%, preferably about 80%. Hydrate form E is deemed to be a dihydrate, whereby some additional water may be absorbed. Polymorph form E can be transformed into polymorph J upon drying under vacuum at moderate temperatures, which may mean between 20° C. and 50° C. at pressures between 0 and 100 mbar. Form E is especially suitable for formulations in semi solid forms because of its stability at high relative humidities.

#### Hydrate Form H

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to

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herein as “form H,” or “hydrate H.” The hydrate form H has a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form H is a hygroscopic monohydrate. The hydrate form H is formed at temperatures below room temperature. Hydrate form H is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form H can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form H exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 15.8 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w), 3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m). FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form H may be obtained by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water, adding then a non-solvent to precipitate a crystalline solid, cooling the obtained suspension and stirring the cooled suspension for a certain time. The crystalline solid is filtered off and then dried under vacuum at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is tetrahydrofuran. Hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and a less amount than that of acetic acid of water, adding a non-solvent and cooling the obtained suspension to temperatures in the range of -10 to 10° C., and preferably -5 to 5° C., and stirring the suspension at said temperature for a certain time. Certain time may mean 1 to 20 hours. The weight ratio of acetic acid to water may be from 2:1 to 25:1 and preferably 5:1 to 15:1. The weight ratio of acetic acid/water to the non-solvent may be from 1:2 to 1:5. Hydrate form H seems to be a monohydrate with a slight excess of water absorbed due to the hygroscopic nature.

#### Hydrate Form O

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form O,” or “hydrate O.” The hydrate form O is formed at temperatures near room temperature. Hydrate form O is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form O can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form O exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m). FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

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Hydrate form O can be prepared by exposure of polymorphic form F to a nitrogen atmosphere containing water vapor with a resulting relative humidity of about 52% for about 24 hours. The fact that form F, which is a slightly hygroscopic anhydrate, can be used to prepare form O under 52% relative humidity suggests that form O is a hydrate, which is more stable than form F under ambient temperature and humidity conditions.

#### Solvate-Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline solvate forms, which shall be described and defined herein as forms G, I, L, M, and N. These solvate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

#### Solvate Form G

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form G," or "hydrate G." The ethanol solvate form G has a ethanol content of approximately 8.0 to 12.5 percent by weight, which suggests that form G is a hygroscopic mono ethanol solvate. The solvate form G is formed at temperatures below room temperature. Form G is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form G can be prepared as a solid powder with a desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form G exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w). FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form G may be obtained by crystallization of L-erythro-tetrahydrobiopterin dihydrochloride dissolved in water and adding a large excess of ethanol, stirring the obtained suspension at or below ambient temperatures and drying the isolated solid under air or nitrogen at about room temperature. Here, a large excess of ethanol means a resulting mixture of ethanol and water with less than 10% water, preferably about 3 to 6%. Ethanolate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at about room temperature to temperatures of 75° C. (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water or in a mixture of water and ethanol, cooling a heated solution to room temperature and down to 5 to 10° C., adding optionally ethanol to complete precipitation, stirring the obtained suspension at temperatures of 20 to 5° C., filtering off the white, crystalline solid and drying the solid under air or a protection gas such as nitrogen at temperatures about room temperature. The process may be carried out in a first variant in dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at about room temperature in a lower amount of water and then adding an excess of ethanol and then stirring the obtained suspension for a time sufficient for phase equilibration. In a second variant, (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be suspended in ethanol, optionally adding a lower amount of water, and heating the suspension and dissolute (6R)-L-erythro-tetrahydrobiopterin

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dihydrochloride, cooling down the solution to temperatures of about 5 to 15° C., adding additional ethanol to the suspension and then stirring the obtained suspension for a time sufficient for phase equilibration.

#### Solvate Form I

It has been found that an acetic acid solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form I," or "hydrate I." The acetic acid solvate form I has an acetic acid content of approximately 12.7 percent by weight, which suggests that form I is a hygroscopic acetic acid mono solvate. The solvate form I is formed at temperatures below room temperature. Acetic acid solvate form I is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form I can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form I exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m)+3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w). FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Acetic acid solvate form I may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water at elevated temperature, adding further acetic acid to the solution, cooling down to a temperature of about 10° C., then warming up the formed suspension to about 15° C., and then stirring the obtained suspension for a time sufficient for phase equilibration, which may last up to 3 days. The crystalline solid is then filtered off and dried under air or a protection gas such as nitrogen at temperatures about room temperature.

#### Solvate Form L

It has been found that a mixed ethanol solvate/hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form L," or "hydrate L." Form L may contain 4% but up to 13% ethanol and 0% to about 6% of water. Form L may be transformed into form G when treated in ethanol at temperatures from about 0° C. to 20° C. In addition form L may be transformed into form B when treated in an organic solvent at ambient temperatures (10° C. to 60° C.). Polymorph form L can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form L exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w). FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Form L may be obtained by suspending hydrate form E at room temperature in ethanol and stirring the suspension at temperatures from 0 to 10° C., preferably about 5° C., for a time sufficient for phase equilibration, which may be 10 to 20 hours. The crystalline solid is then filtered off and dried preferably under reduced pressure at 30° C. or under nitrogen. Analysis by TG-FTIR suggests that form L may contain



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variable amounts of ethanol and water, i.e., it can exist as an polymorph (anhydrate), as a mixed ethanol solvate/hydrate, or even as a hydrate.

#### Solvate Form M

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form M," or "hydrate M." Form M may contain 4% but up to 13% ethanol and 0% to about 6% of water, which suggests that form M is a slightly hygroscopic ethanol solvate. The solvate form M is formed at room temperature. Form M is especially suitable as intermediate and starting material to produce stable polymorph forms, since form M can be transformed into form G when treated in ethanol at temperatures between about -10° to 15° C., and into form B when treated in organic solvents such as ethanol, C3 and C4 alcohols, or cyclic ethers such as THF and dioxane. Polymorph form M can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form M exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs). FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in ethanol and evaporation of the solution under nitrogen at ambient temperature, i.e., between 10° C. and 40° C. Form M may also be obtained by drying of form G under a slight flow of dry nitrogen at a rate of about 20 to 100 ml/min. Depending on the extent of drying under nitrogen, the remaining amount of ethanol may be variable, i.e., from about 3% to 13%.

#### Solvate Form N

It has been found that another solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form N," or "hydrate N." Form N may contain in total up to 10% of isopropanol and water, which suggests that form N is a slightly hygroscopic isopropanol solvate. Form N may be obtained through washing of form D with isopropanol and subsequent drying in vacuum at about 30° C. Form N is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form N can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form N exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83 (w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w). FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

The isopropanol form N may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in 4.0 ml of a mixture of isopropanol and water (mixing volume ratio for example 4:1). To this solution is slowly added isopropanol (IPA, for example about 4.0 ml) and the resulting suspension is cooled to 0° C. and stirred for several hours (e.g., about 10 to 18 hours) at this temperature. The suspension is filtered and the solid residue washed with isopropanol at room temperature. The obtained crystalline material is then dried at ambient temperature (e.g., about 20 to 30° C.) and reduced pressure

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(about 2 to 10 mbar) for several hours (e.g., about 5 to 20 hours). TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water. This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as a non-solvated form containing a small amount of water.

For the preparation of the polymorph forms, there may be used crystallization techniques well known in the art, such as stirring of a suspension (phase equilibration in), precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 100° C. may be applied to form solutions. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Meta-stable polymorphs or pseudo-polymorphic forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

It was surprisingly found that hydrate form D is the most stable form under the hydrates and forms B and D are especially suitable to be used in pharmaceutical formulations. Forms B and D presents some advantages like an aimed manufacture, good handling due to convenient crystal size and morphology, very good stability under production conditions of various types of formulation, storage stability, higher solubility, and high bio-availability. Accordingly, in a method and/or a composition disclosed herein the form of BH4 present in a mixture is preferably a stabilized crystal form of BH4 an is selected from the group consisting of crystal polymorph form A, crystal polymorph form B, crystal polymorph form F, crystal polymorph form J, crystal polymorph form K, crystal hydrate form C, crystal hydrate form D, crystal hydrate form E, crystal hydrate form H, crystal hydrate form O, solvate crystal form G, solvate crystal form I, solvate crystal form L, solvate crystal form M, solvate crystal form N, and combinations thereof. More preferably, the form of BH4 is for use in a composition and method disclosed herein is pharmaceutical composition including polymorph form B and/or hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

The crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be used together with folic acid or tetrahydrofolic acid or their pharmaceutically acceptable salts such as sodium, potassium, calcium or ammonium salts, each alone or additionally with arginine. The weight ratio of crystal forms:folic acids or salts thereof:arginine may be from about 1:10:10 to about 10:1:1.

#### VI. Pharmaceutical Formulations

The formulations described herein are preferably administered as oral formulations. Oral formulations are preferably solid formulations such as capsules, tablets, pills and troches, or liquid formulations such as aqueous suspensions, elixirs and syrups. The various form of BH4 described herein can be directly used as powder (micronized particles), granules, suspensions or solutions, or it may be combined together with other pharmaceutically acceptable ingredients in admixing the components and optionally finely divide them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches, or suspend or dissolve them in carriers for suspensions, elixirs and syrups. Coatings may be applied after compression to form pills.

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Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be for example binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. Nonlimiting examples of binders useful in a composition described herein include gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (polyoxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Nonlimiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate. Nonlimiting examples of lubricants use in a composition described herein include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Surfactants for use in a composition described herein can be anionic, anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid; 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The hydrate form D described herein may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. A syrup or elixir may contain the polymorph described herein,

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sucrose or fructose as sweetening agent a preservative like methylparaben, a dye and a flavoring agent.

Slow release formulations may also be prepared from the polymorph described herein in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg×5 kg body weight) to 3000 mg/day (30 mg/kg×100 kg body weight). Such a dose may be administered in a single dose or it may be divided into multiple doses. While continuous, daily administration is contemplated, it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

As discussed above, the total dose required for each treatment may be administered in multiple doses or in a single dose. The BH4 and the protein compositions may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

As is apparent from the disclosure presented herein, in a broad aspect the present application contemplates clinical application of a combination therapy comprising a first composition that contains a crystallized BH4 formulation, and a second composition that contains a medical protein formulation (e.g., PHENEX or the like). Therefore, the compositions should be formulated into suitable pharmaceutical compositions, i.e., in a form appropriate for in vivo applications in such combination therapies. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals. Preferably, the crystallized BH4 composition may be such that it can be added directly to the preexisting protein formulations used for the treatment of PKU.

One will generally desire to employ appropriate salts and buffers to render the BH4 suitable for uptake. Aqueous compositions of the present invention comprise an effective amount of the BH4 dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions may be administered orally or via injection.

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The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions. In exemplary embodiments, the medical protein formulation may comprise corn syrup solids, high-oleic safflower oil, coconut oil, soy oil, L-leucine, calcium phosphate tribasic, L-tyrosine, L-proline, L-lysine acetate, DATEM (an emulsifier), L-glutamine, L-valine, potassium phosphate dibasic, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-serine, potassium citrate, L-threonine, sodium citrate, magnesium chloride, L-histidine, L-methionine, ascorbic acid, calcium carbonate, L-glutamic acid, L-cystine dihydrochloride, L-tryptophan, L-aspartic acid, choline chloride, taurine, m-inositol, ferrous sulfate, ascorbyl palmitate, zinc sulfate, L-carnitine, alpha-tocopheryl acetate, sodium chloride, niacinamide, mixed tocopherols, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, manganese sulfate, riboflavin, pyridoxine hydrochloride, folic acid, beta-carotene, potassium iodide, phyloquinone, biotin, sodium selenate, chromium chloride, sodium molybdate, vitamin D3 and cyanocobalamin. The amino acids, minerals and vitamins in the supplement should be provided in amounts that provide the recommended daily doses of each of the components.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention include classic pharmaceutical preparations of BH4 which have been discussed herein as well as those known to those of skill in the art. Protein formulas, such as, e.g., Phenex, also are known to those of skill in the art. Administration of these compositions according to the present invention will be via any common route for dietary supplementation. The protein is preferably administered orally, as is the BH4.

The active compounds may be prepared for administration as solutions of free base or pharmacologically acceptable salts in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The BH4 compositions may be prepared as pharmaceutical forms suitable for injectable use. Such compositions include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of

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microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used.

Compositions and methods for producing a stabilized tablet formulation are also disclosed in co-pending U.S. provisional application No. 60/629,189 filed Nov. 17, 2004, the entirety of which is hereby incorporated by reference.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4



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aminosalicylic acid, 2 phenoxybenzoic acid, 2 acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzenesulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2 sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data. The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey ducks and geese.

## VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be

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made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## Example 1

## Clinical Evaluation with 6R-Tetrahydrobiopterin

The following example provides guidance on the parameters to be used for the clinical evaluation BH4 in the therapeutic methods of the present invention. As discussed herein throughout, BH4 will be used in the treatment of HPA including HPA, mild phenylketonuria (PKU) and classic PKU. Clinical trials will be conducted which will provide an assessment of daily oral doses of BH4 for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 6 weeks to collect sufficient safety information for 30 evaluable patients.

The initial dose for the trials will vary from about 10 to about 20 mg/kg. In the event that this dose does not produce a reduction in excess plasma phenylalanine (Phe) levels in a patient, or produce a significant direct clinical benefit measured as an ability to increase daily oral Phe intake without increases in plasma Phe levels, the dose should be increased as necessary, and maintained for an additional minimal period of, but necessarily limited to, 6 weeks to establish safety and to evaluate further efficacy. Lower doses, e.g., doses of between 5 to 10 mg/kg also are contemplated.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (kidney and liver function), urinalysis, and CBC with differential. In addition, other parameters including the reduction in levels of blood Phe levels, neuropsychological and cognitive testing, and global assessments also will be monitored. The present example also contemplates the determination of pharmacokinetic parameters of the drug in the circulation, and general distribution and half-life of 6R-BH4 in blood. It is anticipated that these measures will help relate dose to clinical response.

## Methods

Patients who have elevated levels of plasma Phe will undergo a baseline a medical history and physical exam, neuropsychological and cognitive testing, a standard set of clinical laboratory tests (CBC, Panel 20, CH50, UA), levels of urinary pterins, dihydropteridine reductase (DHPR) levels, and a fasting blood (plasma) panel of serum amino acids. The proposed human dose of 10 to about 20 mg/kg BH4 will be administered divided in one to three daily doses. The patient will be followed closely with weekly visits to the clinic. Patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Enrolled patients will be randomized to receive BH4 or a placebo. After an initial two to four-week period all study participants will be placed on a controlled diet with a limited Phe intake for a total of four to six weeks. After completing the first two to four weeks on dietary restriction, all study participants will be crossed-over in their randomization and will followed for an additional two to four weeks. The blood levels and other biochemical parameters will be followed closely at the end of each period. Evaluation of neuropsychological outcomes will include measurements of sustained attention; working memory; and ability to perform complex

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operations. Patients who complete the trial, and who benefited from therapy by showing a beneficial decrease plasma Phe levels, will be offered continued BH4 therapy thorough an extended protocol for as long as safety and efficacy conditions warrant it, or until BLA approval.

#### Diagnosis and Inclusion/Exclusion Criteria

The patient may be male or female, aged twelve years or older with a documented diagnosis of HPA or mild PKU confirmed by genetic testing and evidence of elevated Phe levels in blood. The study will include HPA or PKU patients who do not accurately follow dietary control. Female patients of childbearing potential must have a negative pregnancy test (urine  $\beta$ -hCG) just prior to each dosing and must be advised to use a medically accepted method of contraception throughout the study. A patient will be excluded from this study if the patient has evidence of a primary BH4 deficiency, has previously received multiple doses of BH4 for more than 1 week of treatment; is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

#### Dose, Route and Regimen

Patients will receive BH4 at a dose of 5-10 mg/kg per day. In the event that Phe blood levels are not decreased by a reasonable amount and no clinical benefit is observed, the dose will be increased as necessary. Dose escalation will occur only after all patients have undergone at least 2 weeks of therapy. The daily BH4 dosage will be administered orally as liquid, powder, tablets or capsules. The total daily dose may be given as a single dose or perhaps divided in two or three daily doses. The patients will be monitored clinically as well as for any adverse reactions. If any unusual symptoms are observed, study drug administration will be stopped immediately, and a decision will be made about study continuation.

#### Dietary Intervention

Following the initial randomization and two week treatment period, all study participants will undergo dietary counseling and will follow a standard Phe-restricted diet complemented with Phe-specific medical foods for a total of four to six weeks. Diets will be managed at home and dietary intake will be recorded in daily logs. Analyses of the intakes of nutrients and medical foods and the percent of Recommended Dietary Intakes (RDI) will be compared among the treatment groups.

#### BH4 Safety

BH4 therapy will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

#### Example 2

##### Preparation of Stabilized Crystallized form of BH4

U.S. Provisional Patent Application Ser. No. 60/520,377, entitled "Polymorphs of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride" filed on Nov. 17, 2003 in the name of Applicants Rudolf MOSER, of Schaffhausen, Switzerland and Viola GROEHN of Dachsen, Switzerland and assigned Merck-Eprova internal reference number 216, and U.S. patent application Ser. No. 10/990,316, entitled "Polymorphs

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of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride" filed concurrently herewith on Nov. 17, 2004 in the name of Applicants Rudolf MOSER, of Schaffhausen, Switzerland and Viola GROEHN of Dachsen, Switzerland and assigned Merck-Eprova internal reference number 216/US CIP (both of the Moser et al. applications are collectively referred to herein as the "Moser Applications" and both are incorporated herein by reference in their entireties. The examples of that specification describe X ray and Raman spectra studies to characterize the polymorphs of BH4. Each of the BH4 compositions of that application may be used in the treatment methods described herein. The following description provides additional background and a brief characterization of some of those exemplary compositions.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride (see the Moser Applications) indicated that the compound may possess polymorphic forms. The continued interest in this area requires an efficient and reliable method for the preparation of individual polymorphs of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and controlled crystallization conditions to provide polymorphs, which are preferably stable and easily to handle and to process in the manufacture and preparation of formulations.

Crystallization techniques well known in the art for producing drug crystals are used to prepare the polymorph forms. Such techniques include, but are not limited to, techniques such as suspension, precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions of the BH4 may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 150° C. may be applied to form solutions of the drug. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Metastable polymorph or pseudo-polymorph forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

As discussed in the Moser Applications, the polymorph form may be obtained by crystallization of the BH4 from polar solvent mixtures. The Moser Applications also describes a process for the preparation of polymorph form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolution, optionally at elevated temperatures, of a solid lower energy form than the claimed form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a polar solvent mixture, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals.

Dissolution may be carried out at room temperature or up to 70° C., More preferably the dissolution is carried out at temperatures up to 50° C. The starting material may be added to the final solvent mixture for dissolution, or alternatively the starting material first may be dissolved in water and other solvents may then be added both or one after the other solvent. The solution of the BH4 is preferably stirred. Cooling may mean temperatures down to -80° C., preferably down to -40° C. to 0° C. In some embodiments, in order to initiate the crystallization of the BH4 polymorph, the solution may be seeded. Suitable seeds may include a portion of the polymorph form from another batch of crystals, or crystals having a similar or identical morphology. After isolation, the crystalline form can be washed with acetone or tetrahydrofuran and dried using techniques commonly used for drying drug crystals.

The polymorph forms of BH4 described in the Moser Applications are a very stable crystalline form of the drug.



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The polymorph form can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders this polymorph form especially feasible for pharmaceutical application. The stability of the polymorph form of BH4 was determined after the BH4·2HCl (the polymorph form) had been stored for 8 months in a minigrip bag at 40° C. and 75% relative humidity. Quality was checked in different intervals throughout the 8 month period by HPLC. After 8 months, the quality and stability of the polymorph was surprisingly similar to the stability seen at time zero:

	0 months (at the beginning)	after 1 week	after 1 month	after 3 months	after 8 months
HPLC [% area]	98.4	99.4	98.3	99.1	98.1

Accordingly, the Moser Applications provides descriptions of a pharmaceutical compositions comprising a polymorph form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent. Such compositions will be useful in the therapeutic methods described herein.

In addition to the Moser Applications, those of skill in the art also are referred to U.S. Pat. Nos. 6,596,721; 6,441,168; and 6,271,374 which describe various methods and compositions for producing stable crystalline salts of 5-methyltetrahydrofolic acid and methods and compositions for producing stable forms of 6R tetrahydrofolic acid and methods and compositions for producing stable forms of 6S and 6R tetrahydrofolic acid. Each of these patents are incorporated herein by reference in their entirety as generally teaching methods of producing crystalline forms of agents and techniques for characterizing such agents. Such methods may be used in producing stable forms of BH4 for use as pharmaceutical compositions in the treatment methods taught herein.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## Example 3

Administration of Tetrahydrobiopterin to Humans  
with Elevated Serum Phe Levels

An open label, single and multiple dose study was conducted in a total of 20 patients to demonstrate the safety and efficacy of tetrahydrobiopterin in humans with elevated blood levels of phenylalanine (>600 µmol/L). Criteria for inclusion in the study included (1) baseline blood Phe levels of >600 µmol/L, (2) age of at least 8 years. Criteria for exclusion from

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the study included (1) pregnancy or breastfeeding, (2) concurrent diseases or conditions that require medication or treatment, (3) concurrent treatment with any drug known to inhibit folate synthesis, and (4) treatment with any investigational drug within 30 days. Each of the patients also was identified as having a mutation in the phenylalanine hydroxylase (PAH) gene. Study subjects underwent baseline assessments, including medical history with assessment of phenylketonuria (PKU) or hyperphenylalaninemia (HPA) related signs and symptoms, physical examinations, vital signs, serum amino acid (i.e., phenylalanine, tyrosine, and tryptophan) blood levels, and routine laboratory tests (chemistry, hematology, and urinalysis) before inclusion in the study.

The drug tested was (6R)-5,6,7,8-tetrahydrobiopterin, also known as 2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-3H-pteridin-4-one tetrahydrobiopterin, or sapropterin (BH4 or 6R-BH4. The drug was obtained in 10 mg or 50 mg oral tablets from Schircks Laboratories, Switzerland (product no. 11.212 (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride). The half-life of the Schircks 6R-BH4 dihydrochloride salt is approximately 3.5 hours.

Drugs known to inhibit folate synthesis such as bactrim, methotrexate, or 5-FU were not permitted to be administered during the study. Before initiation of 6R-BH4 dosing, a 7 day washout period was required for any drugs known to inhibit folate synthesis. No investigational drugs were permitted to be taken during study participation or within 30 days prior to study enrollment.

Within a maximum of 4 weeks following the completion of baseline assessments, eligible subjects began the first stage of the study. Single ascending doses of 10 mg/kg, 20 mg/kg and 40 mg/kg of 6R-BH4 were administered orally, with a washout period of at least 7 days between each dose, and subjects were monitored 24 hours after each dose. Subjects underwent a safety assessment and blood amino acid (i.e., phenylalanine, tyrosine, and tryptophan) level measurements before and 24 hours after each 6R-BH4 dose. Blood pressure was measured 30 minutes and 1 hour after each dose. Safety assessments included physical examinations, vital signs, serial assessment of PKU or HPA related signs and symptoms, recording of adverse events, and monitoring of changes in laboratory parameters (chemistry, hematology, and urinalysis). Subjects were instructed to continue their usual diet without any modification, and to record daily intake of food and beverages throughout the study.

After the first stage of the study was completed, subjects entered the second stage of the study, during which they received 10 mg/kg of 6R-BH4 daily in an oral dosage form, for a total of 7 days. After a washout period of at least 7 days, each subject received 20 mg/kg of 6R-BH4 daily for a total of 7 days. During the second stage of the study, subjects were monitored before dosing, at 24 and 72 hours after first dose, and on the 7th day of dosing at each of the two dose levels. Monitoring included a safety assessment as described above, measurement of serum blood amino acid (i.e., phenylalanine, tyrosine, and tryptophan) levels and evaluation of phenylalanine and tyrosine oral intake. Subjects were instructed to continue their usual diet without any modification, and to record daily intake of food and beverages throughout the study.

After a single dose of 6R-BH4 (10 mg/kg), blood Phe declined 10%±0.26% from baseline. Single doses of 6R-BH4 at 20 mg/kg and 40 mg/kg showed mean declines of 17%±0.28% and 27%±0.25% respectively. The reduction in blood Phe levels appeared to be dose dependent.

FIG. 16 shows mean blood phenylalanine level after 10 and 20 mg/kg 6R-BH4 daily for 7 days, in the 14 of 20 patients

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who responded to treatment. For the purposes of this study, a decline in blood Phe levels of 30% was considered to be “responsive”, although patients who exhibit less of a decline would still benefit from BH4 treatment. The seven-day trial showed a sustained decrease in blood Phe concentration in 70% of the patients (14/20) taking 20 mg/kg. Of those 14 patients, 10 (71%) responded favorably to 10 mg/kg/day. Blood tyrosine was observed to increase in some but not all patients; some patients had increases of >80% from baseline tyrosine levels. The individual blood Phe responses to multiple doses of 10 mg/kg BH4 are shown in 11 adults (FIG. 17) and 9 children (FIG. 19). The individual blood Phe responses to multiple doses of 20 mg/kg BH4 are shown in 11 adults (FIG. 18) and in 9 children (FIG. 20).

Thus, a single-dose loading test was inadequate to identify patients who responded to BH4 treatment with a reduction in blood Phe level of 30% or more. A 7-day loading test successfully identified a high percentage of responsive patients. The 20 mg/kg, 7-day loading test with 6R-BH4 identified 70% of the PKU patients that responded to 20 mg/kg of BH4. Of the 14 responders, 71% also showed a 30% or greater reduction in blood Phe level with the lower dose of 10 mg/kg 6R-BH4.

The references cited herein throughout, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.

What is claimed is:

1. A method for treating a subject with hyperphenylalaninemia due to tetrahydrobiopterin-responsive phenylketonuria, comprising administering to said subject a therapeutically effective total daily dosage of tetrahydrobiopterin (BH4) or pharmaceutically acceptable salt thereof, wherein the administering is multiday, oral, and only once per day, and wherein the administering does not achieve controlled release of the BH4 in the gastrointestinal tract.

2. The method of claim 1, wherein the administering is for at least 7 days.

3. The method of claim 1, wherein the subject is administered BH4 for at least 2 weeks.

4. The method of claim 3, wherein said subject is administered BH4 for at least 6 weeks.

5. The method of claim 1, wherein said subject suffers from severe phenylketonuria, moderate phenylketonuria, or mild phenylketonuria.

6. The method of claim 5, wherein said subject suffers from severe phenylketonuria.

7. The method of claim 5, wherein said subject suffers from mild phenylketonuria.

8. The method of claim 5, wherein said subject has been diagnosed as having a mutant phenylalanine hydroxylase (PAH).

9. The method of claim 8, wherein said mutant PAH comprises a mutation in the catalytic domain of PAH.

10. The method of claim 8, wherein said mutation comprises one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M, E390G, A395P, P407S, and Y414C.

11. The method of claim 5, wherein said subject has a plasma phenylalanine concentration of greater than 180  $\mu$ M prior to treatment with BH4.

12. The method of claim 5, wherein said subject has a plasma phenylalanine concentration of greater than 600  $\mu$ M prior to treatment with BH4.

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13. The method of claim 5, wherein said subject has a plasma phenylalanine concentration of greater than 1000  $\mu$ M prior to treatment with BH4.

14. The method of claim 5, wherein said subject has a plasma phenylalanine concentration of greater than 1200  $\mu$ M prior to treatment with BH4.

15. The method of claim 5, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 600  $\mu$ M.

16. The method of claim 5, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 500  $\mu$ M.

17. The method of claim 5, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 360  $\mu$ M.

18. The method of claim 5, wherein said BH4 is administered in a daily dose of between about 1 mg/kg to about 30 mg/kg.

19. The method of claim 5, wherein said BH4 is administered in a daily dose of between about 5 mg/kg to about 30 mg/kg.

20. The method of claim 5, wherein said BH4 is administered as a crystallized form stable for at least 3 months at 40° C. and 75% relative humidity.

21. The method of claim 20, wherein said crystallized form of BH4 comprises at least 99.5% pure (6R)-5,6,7,8-tetrahydrobiopterin.

22. The method of claim 5, further comprising administering to said subject a protein-restricted diet.

23. The method of claim 22, wherein said protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 600 mg per day.

24. The method of claim 22, wherein said protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 300 mg per day.

25. The method of claim 22, wherein said subject is a pregnant female.

26. The method of claim 22, wherein said subject is an infant between the ages of 0 and 3 years of age.

27. The method of claim 22, wherein said subject is a female of child-bearing age that is contemplating pregnancy.

28. The method of claim 20, wherein said crystallized form of BH4 comprises purified polymorph B, wherein polymorph B, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 8.7 (vs), 5.63 (m), 4.76(m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w).

29. The method of claim 20, wherein said crystallized form of BH4 comprises purified polymorph A, wherein polymorph A, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 15.5 (vs), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m), 3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w).

30. The method of claim 20, wherein said crystallized form of BH4 comprises purified polymorph F, wherein polymorph F, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 17.1 (vs), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w),

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3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 2.96 (m).

31. The method of claim 20, wherein said crystallized form of BH4 comprises purified polymorph J, wherein polymorph J, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs).

32. The method of claim 20, wherein said crystallized form of BH4 comprises purified polymorph K, wherein polymorph K, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m).

33. The method of claim 20, wherein said crystallized form of BH4 comprises purified hydrate C, wherein hydrate C, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m).

34. The method of claim 20, wherein said crystallized form of BH4 comprises purified hydrate D, wherein hydrate D, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w).

35. The method of claim 20, wherein said crystallized form of BH4 comprises purified hydrate E, wherein hydrate E, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w).

36. The method of claim 20, wherein said crystallized form of BH4 comprises purified hydrate H, wherein hydrate H, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 8.6 15.8 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w), 3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m).

37. The method of claim 20, wherein said crystallized form of BH4 comprises purified hydrate O, wherein hydrate O, as a hydrochloride salt, exhibits an X-ray powder diffraction

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pattern with the following characteristic peaks expressed in d-values(A): 15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m).

38. The method of claim 20, wherein said crystallized form of BH4 comprises purified solvate G, wherein solvate G, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w).

39. The method of claim 20, wherein said crystallized form of BH4 comprises purified solvate I, wherein solvate I, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m), 3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w).

40. The method of claim 20, wherein said crystallized form of BH4 comprises purified solvate L, wherein solvate L, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w).

41. The method of claim 20, wherein said crystallized form of BH4 comprises purified solvate M, wherein solvate M, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs).

42. The method of claim 20, wherein said crystallized form of BH4 comprises purified solvate N, wherein solvate N, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83 (w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w).

43. The method of claim 1, wherein the BH4 is administered in a daily dose of about 10 mg/kg or more.

44. The method of claim 2, wherein the BH4 is administered in a daily dose of about 10 mg/kg or more.

45. The method of claim 3, wherein the BH4 is administered in a daily dose of about 10 mg/kg or more.

46. The method of claim 4, wherein the BH4 is administered in a daily dose of about 10 mg/kg or more.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

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DATED : July 28, 2009  
INVENTOR(S) : Daniel I. Oppenheimer et al.

Page 1 of 1

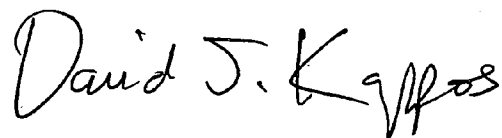
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

At field (73), "Biomarin" should be -- BioMarin --.

Signed and Sealed this

Twenty-third Day of March, 2010

A handwritten signature in black ink that reads "David J. Kappos". The signature is written in a cursive, flowing style with a large, stylized 'D' and 'K'.

David J. Kappos  
*Director of the United States Patent and Trademark Office*

# EXHIBIT C



US007612073B2

(12) **United States Patent**  
**Oppenheimer et al.**(10) **Patent No.:** **US 7,612,073 B2**(45) **Date of Patent:** **\*Nov. 3, 2009**(54) **METHODS OF ADMINISTERING  
TETRAHYDROBIOPTERIN, ASSOCIATED  
COMPOSITIONS, AND METHODS OF  
MEASURING**

WO WO 2005/049000 6/2005

## OTHER PUBLICATIONS

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CA (US); **Alejandro Dorenbaum**, Mill  
Valley, CA (US)(73) Assignee: **Biomarin Pharmaceutical Inc.**, Novato,  
CA (US)(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 0 days.This patent is subject to a terminal dis-  
claimer.(21) Appl. No.: **12/329,838**(22) Filed: **Dec. 8, 2008**(65) **Prior Publication Data**

US 2009/0076014 A1 Mar. 19, 2009

**Related U.S. Application Data**(63) Continuation of application No. PCT/US2008/  
060041, filed on Apr. 11, 2008.(60) Provisional application No. 60/922,821, filed on Apr.  
11, 2007, provisional application No. 61/019,753,  
filed on Jan. 8, 2008.(51) **Int. Cl.****A01N 43/58** (2006.01)**A61K 31/50** (2006.01)**A01N 43/60** (2006.01)**A61K 31/495** (2006.01)(52) **U.S. Cl.** ..... **514/249**(58) **Field of Classification Search** ..... **514/249**  
See application file for complete search history.(56) **References Cited**

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Pediatr.*, (2008), doi:10.1016/j.peds.2008.11.040.*Primary Examiner*—Yong S Chong(74) *Attorney, Agent, or Firm*—Marshall, Gerstein & Borun  
LLP(57) **ABSTRACT**

The present invention is directed to treatment methods of  
administering tetrahydrobiopterin, including in oral dosage  
forms, in intravenous formulations, and with food. Also dis-  
closed herein are biopterin assays for measuring the amount  
of biopterin and metabolites of biopterin in a sample.

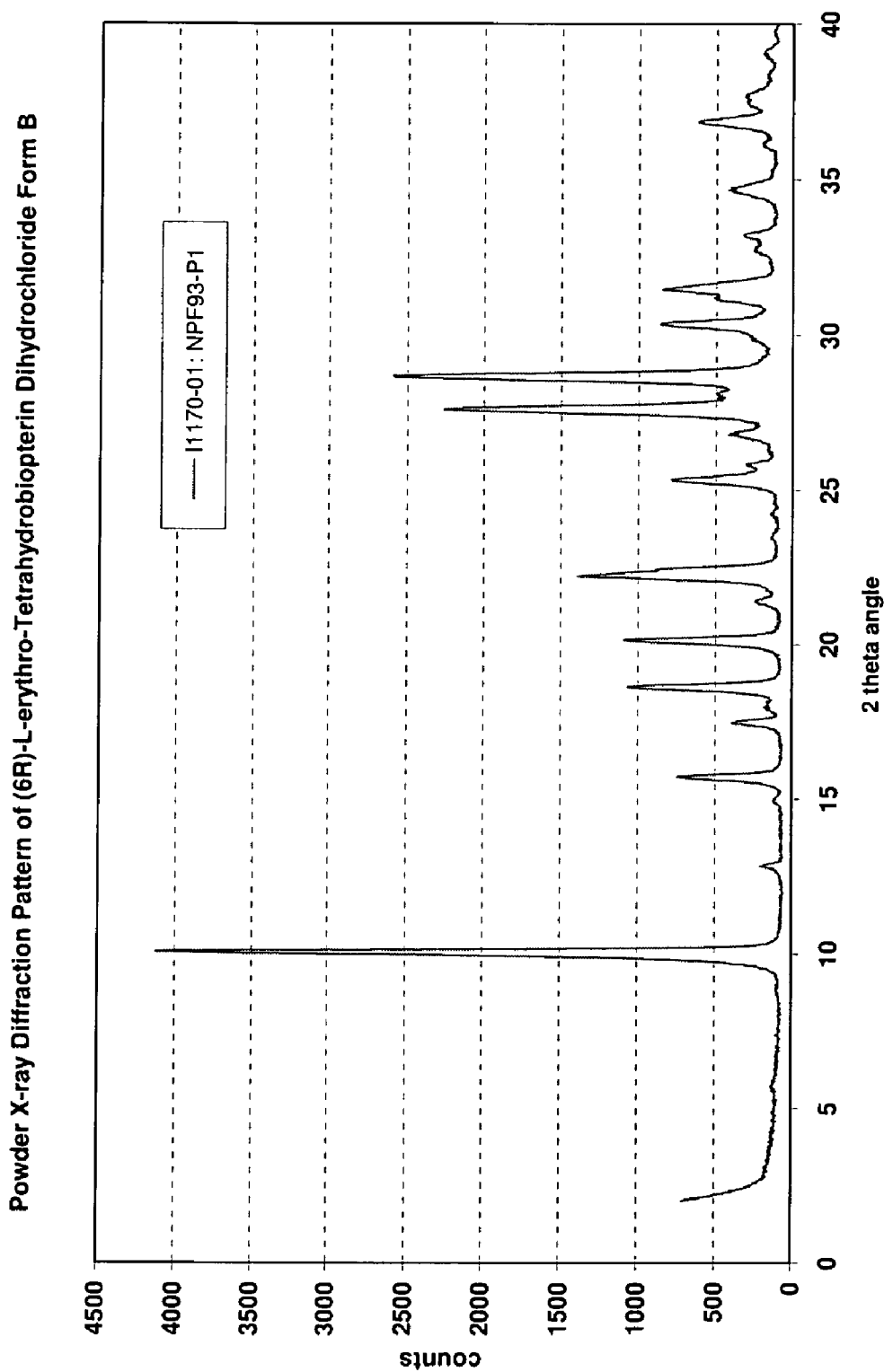
**11 Claims, 38 Drawing Sheets**

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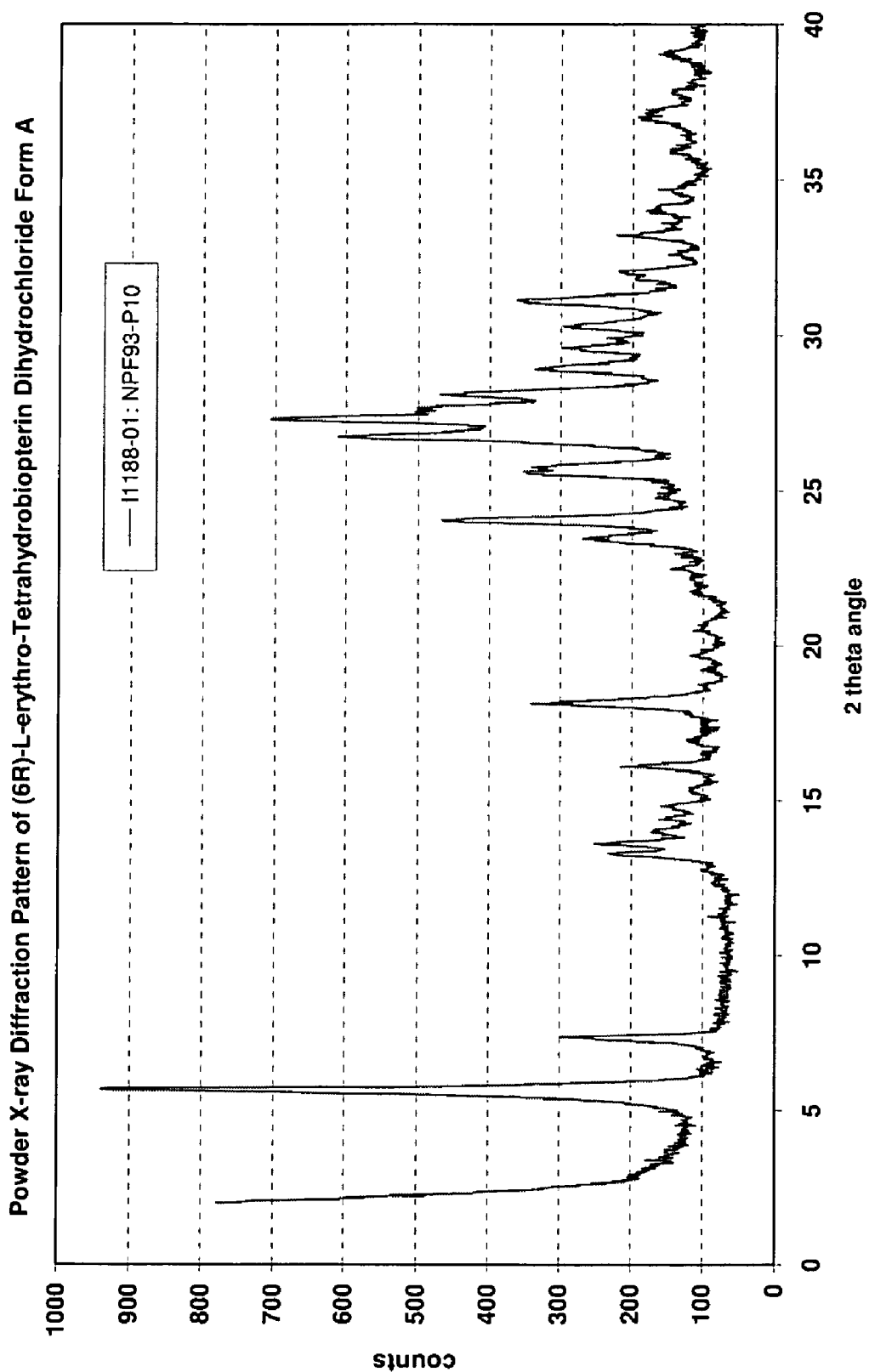
**Figure 1**

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**Figure 2**

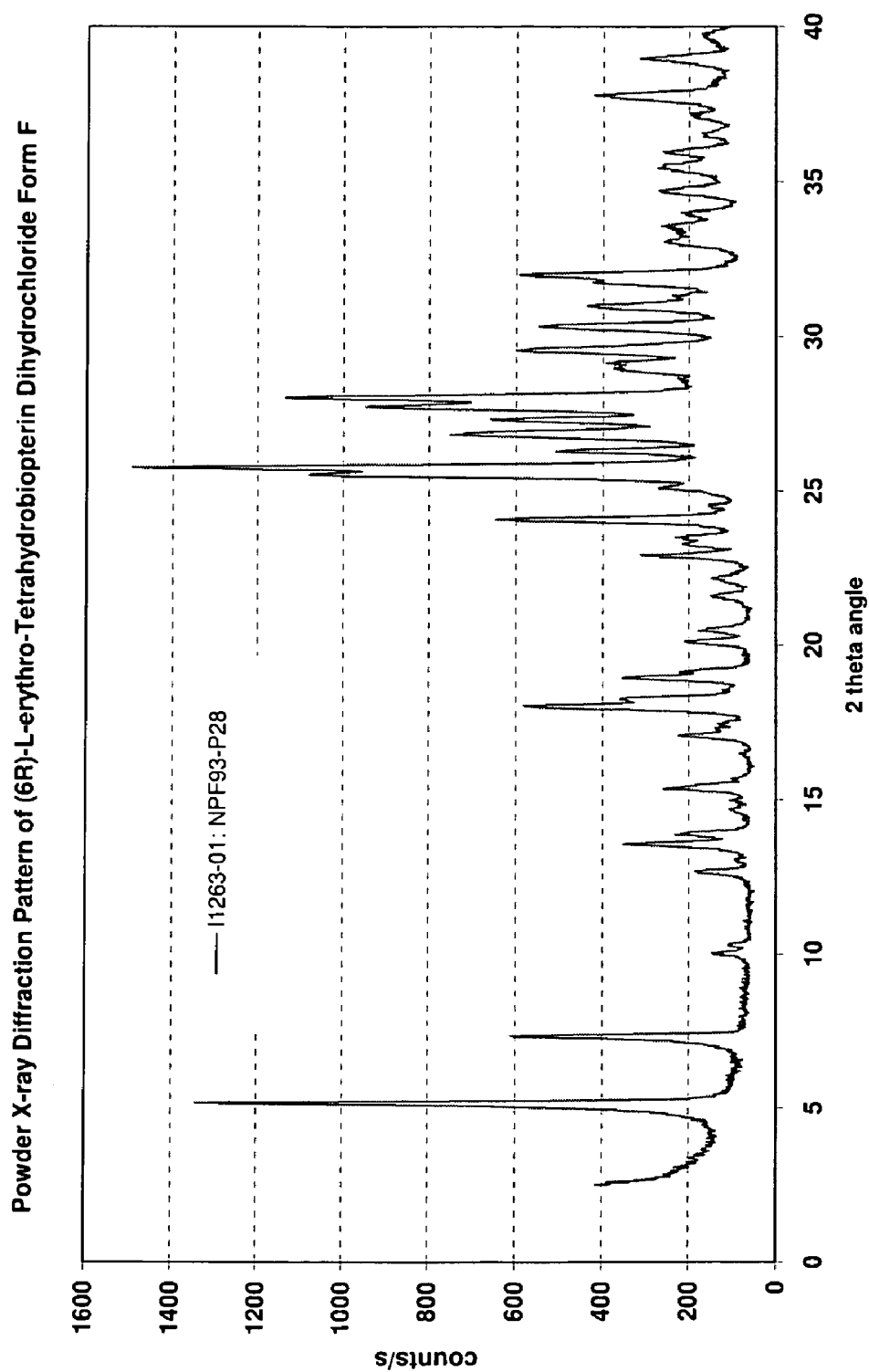


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**Figure 3**

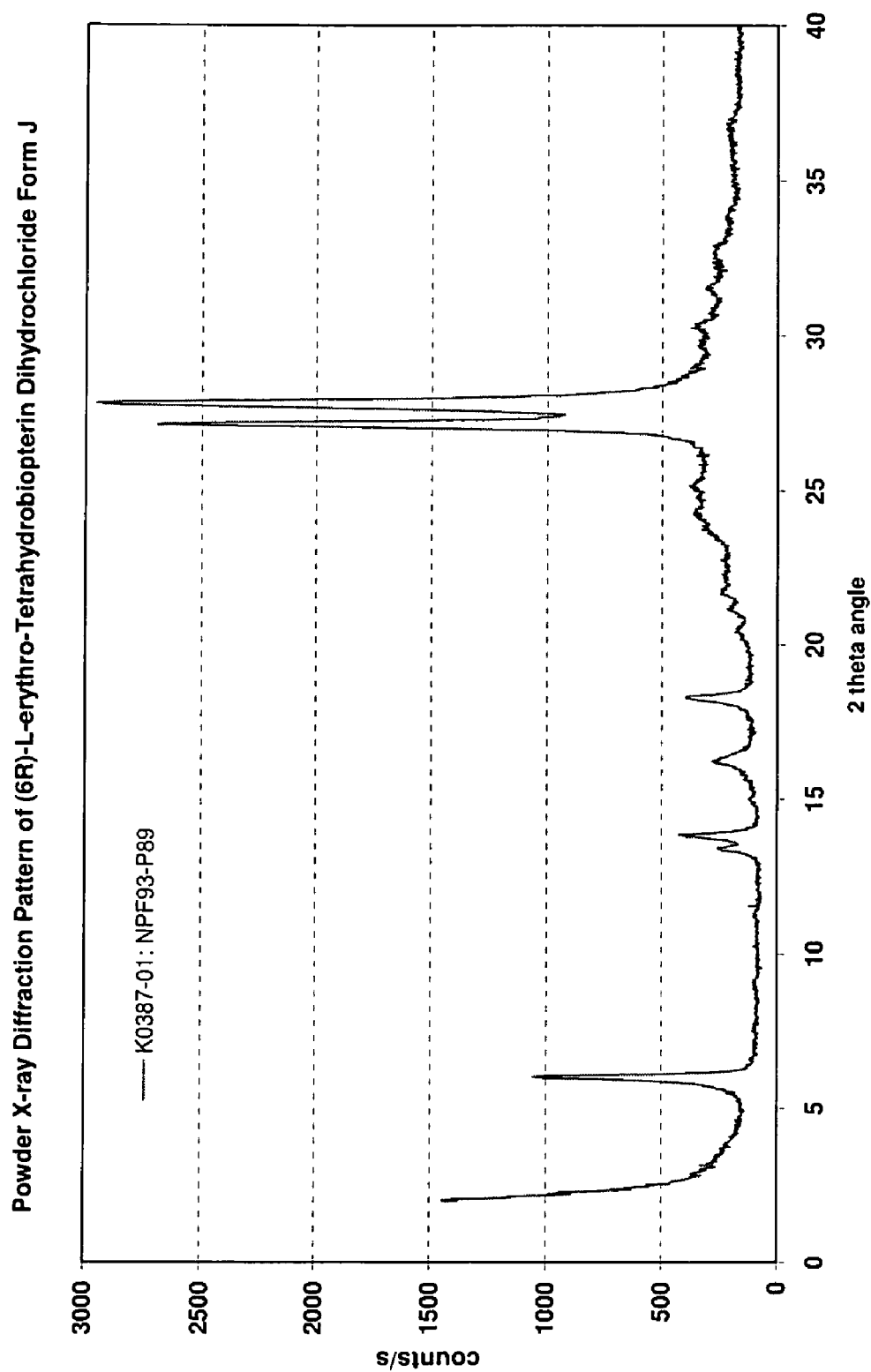
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Figure 4



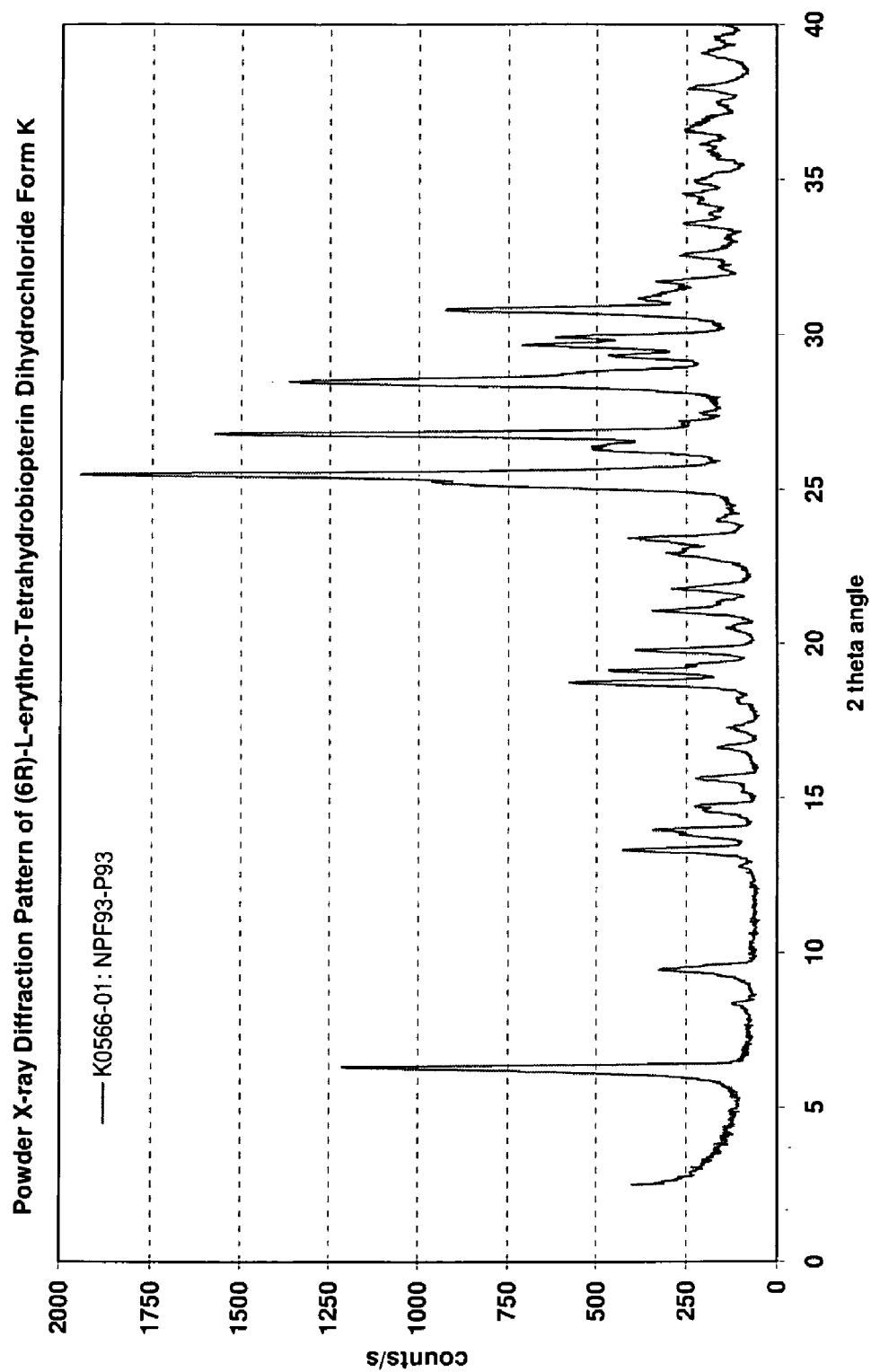
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Figure 5

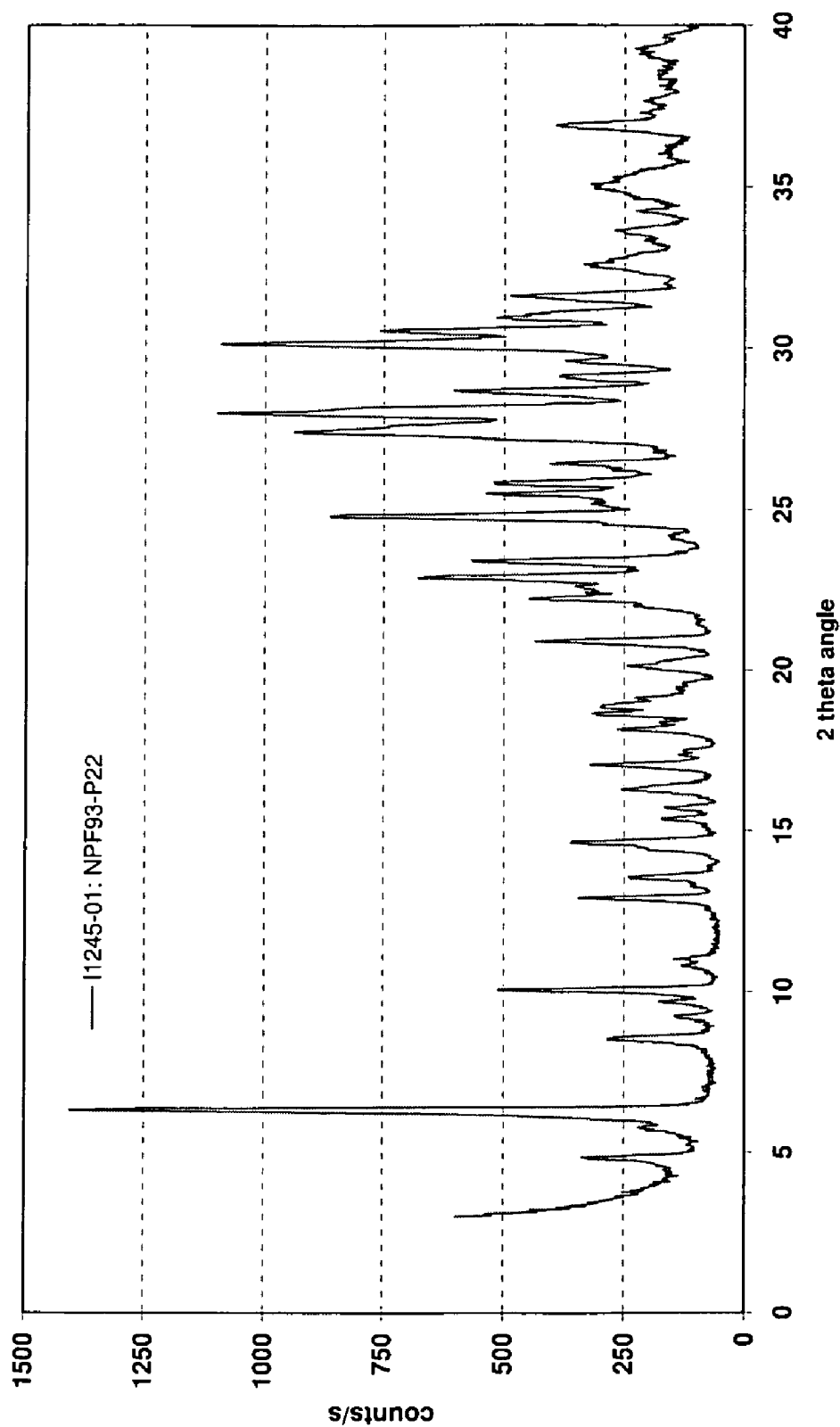


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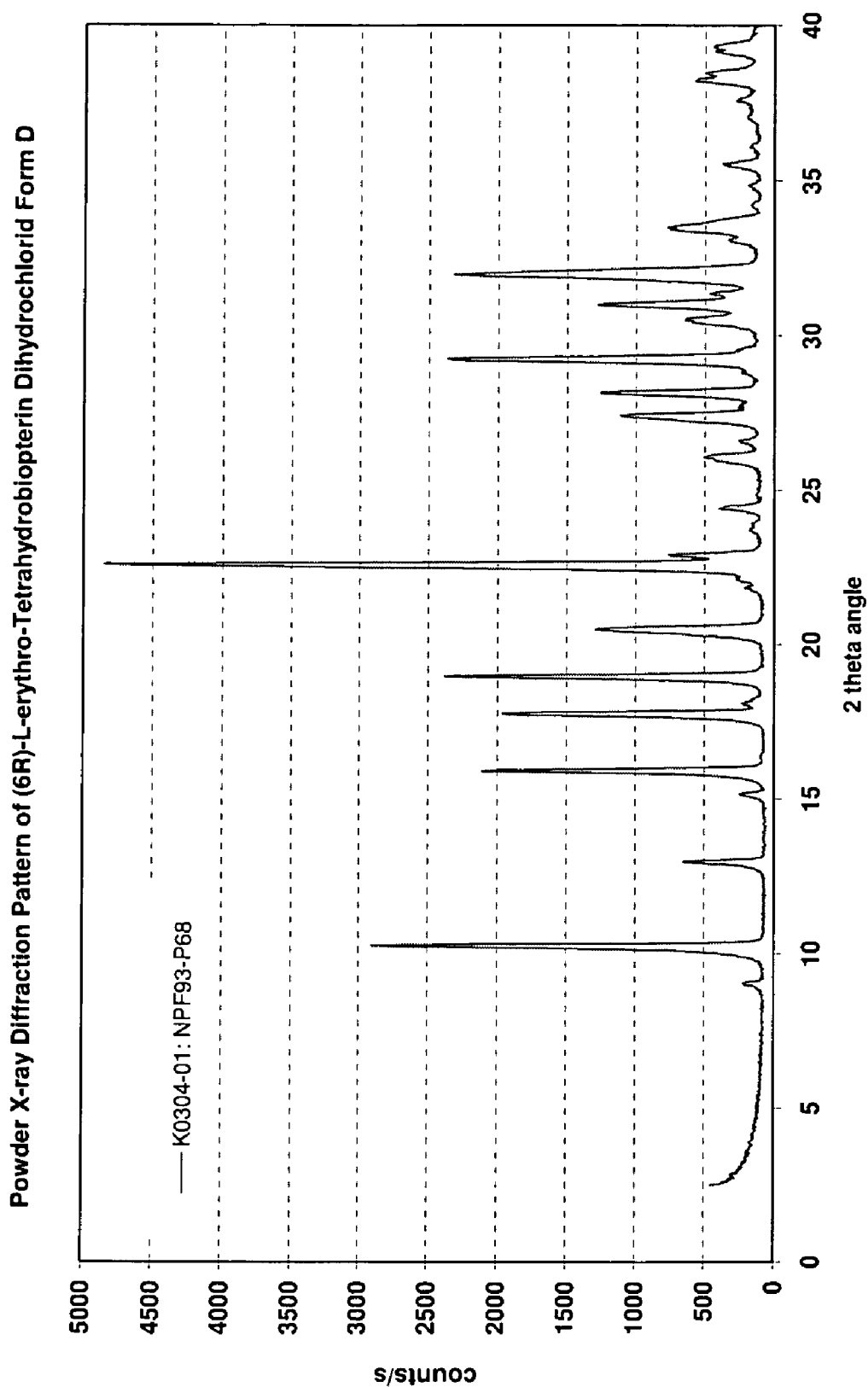
**Figure 6****Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochlorid Form C**

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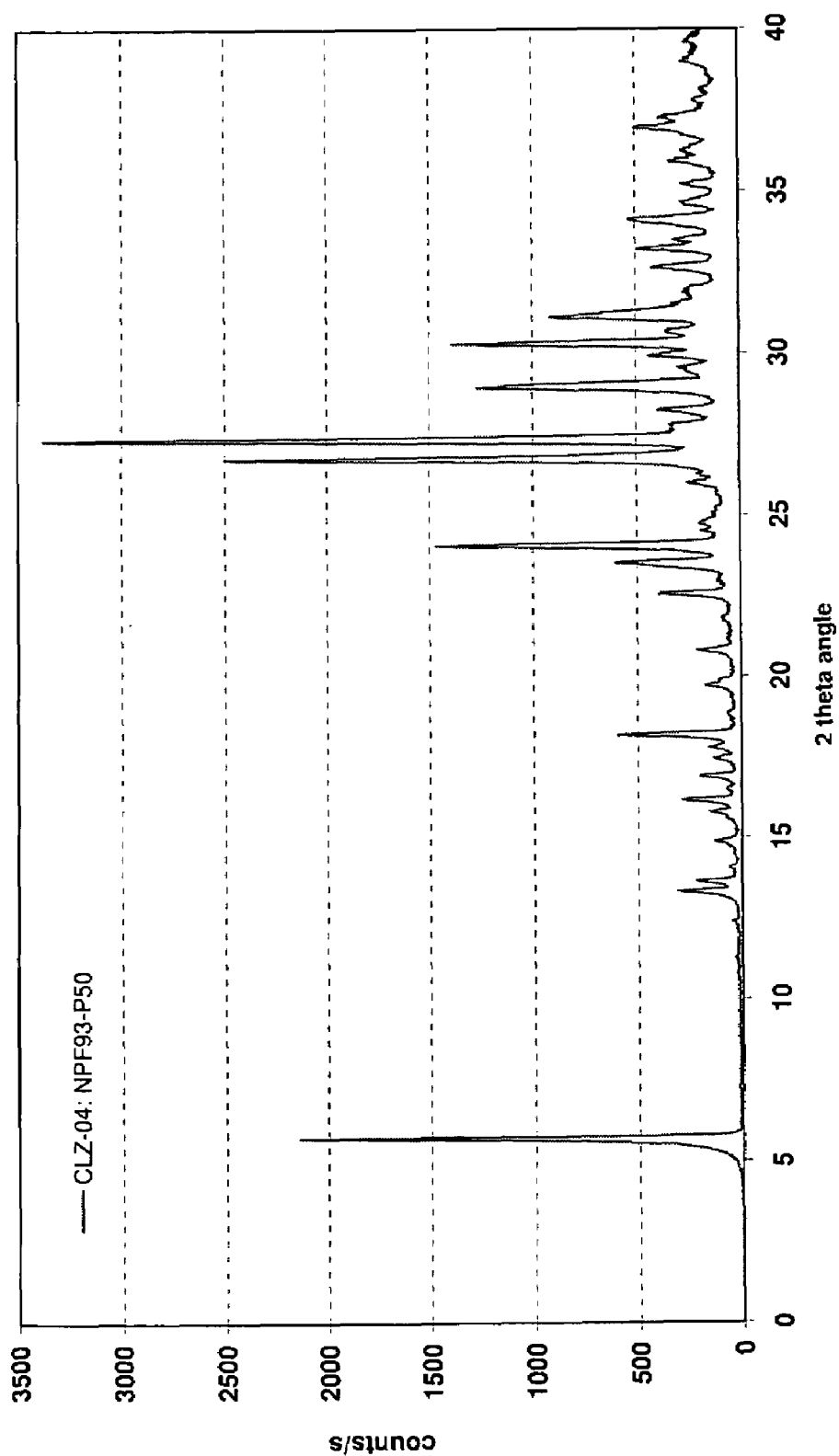
**Figure 7**

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**Figure 8****Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form E**

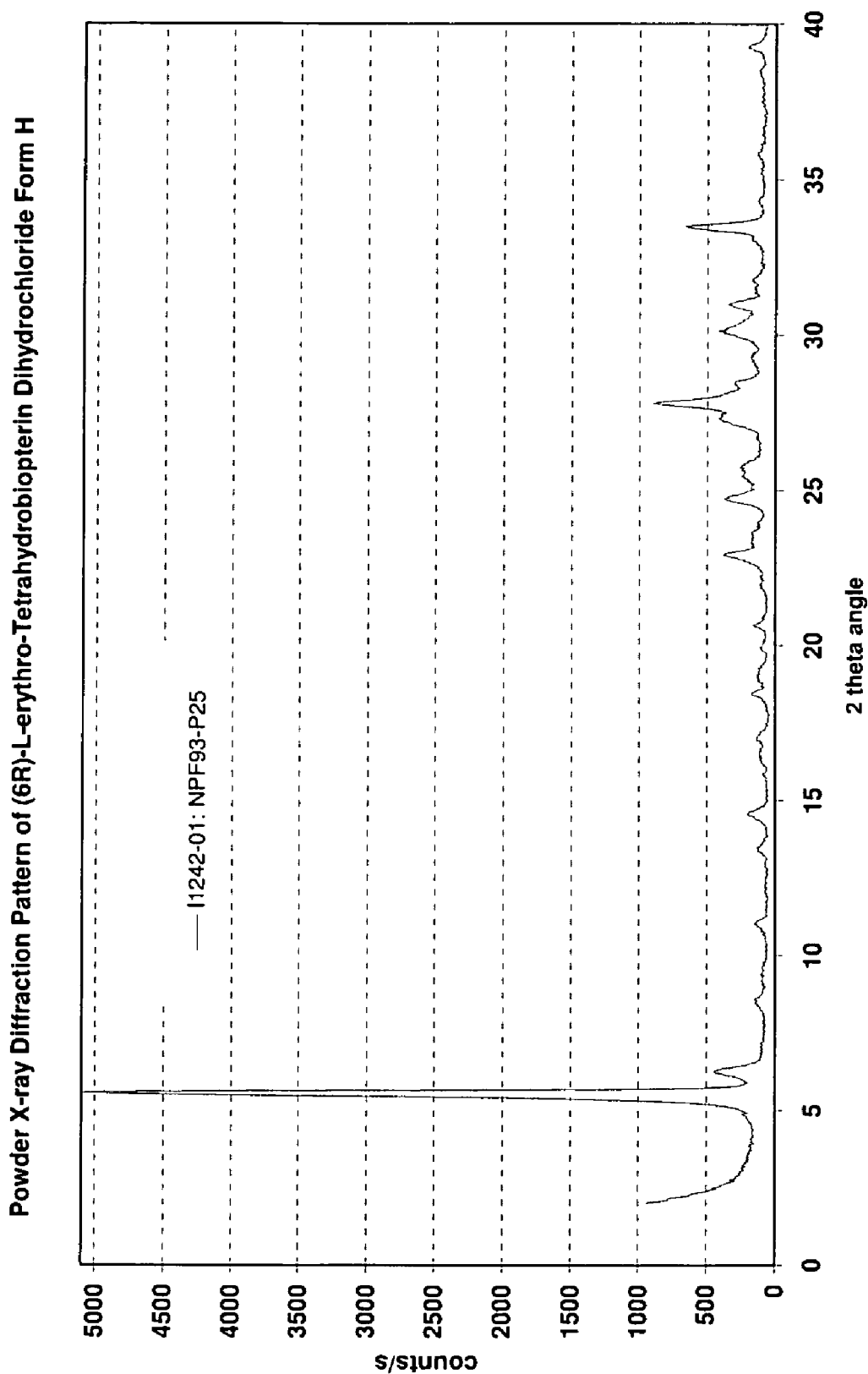
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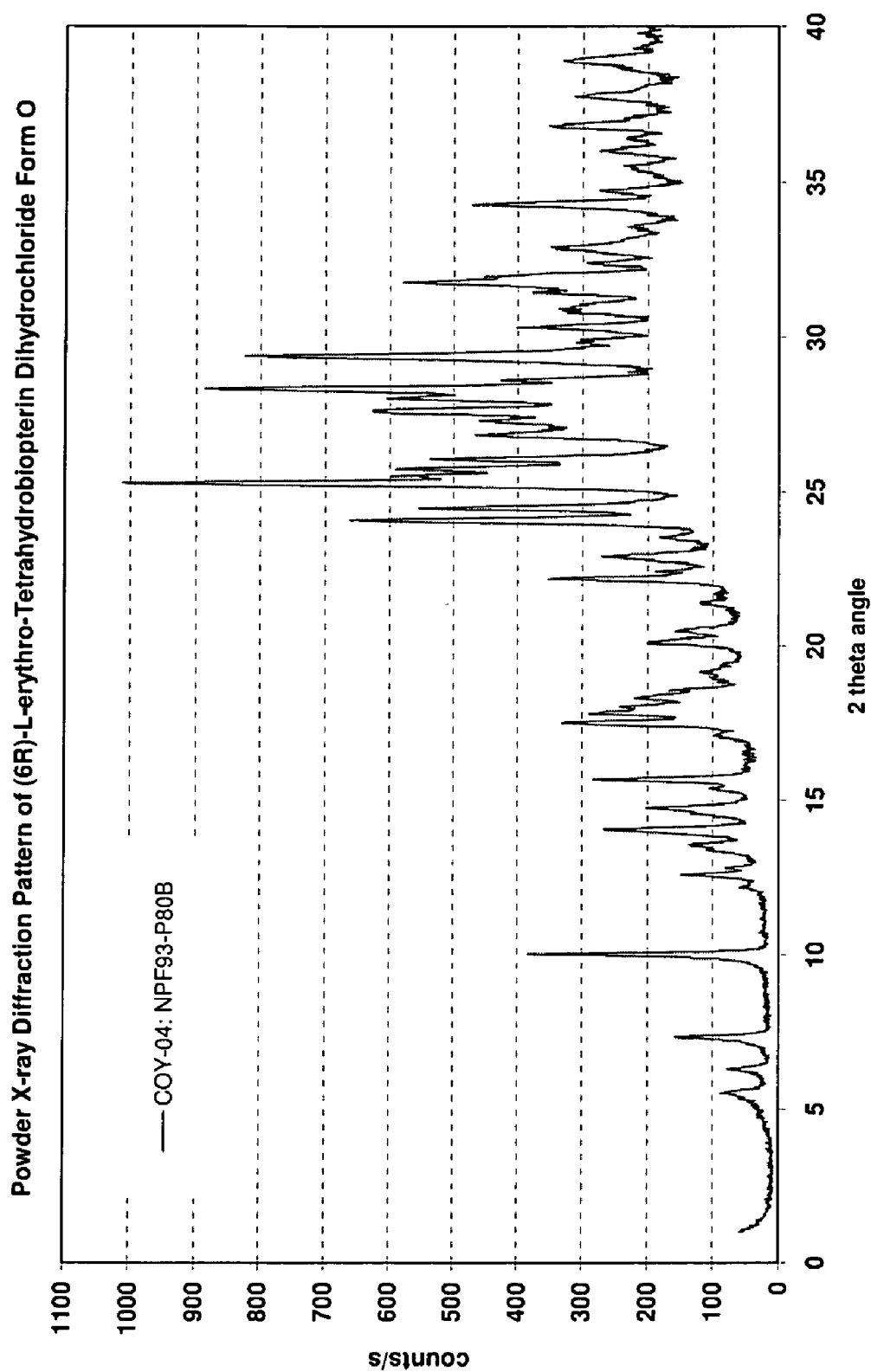
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Figure 9





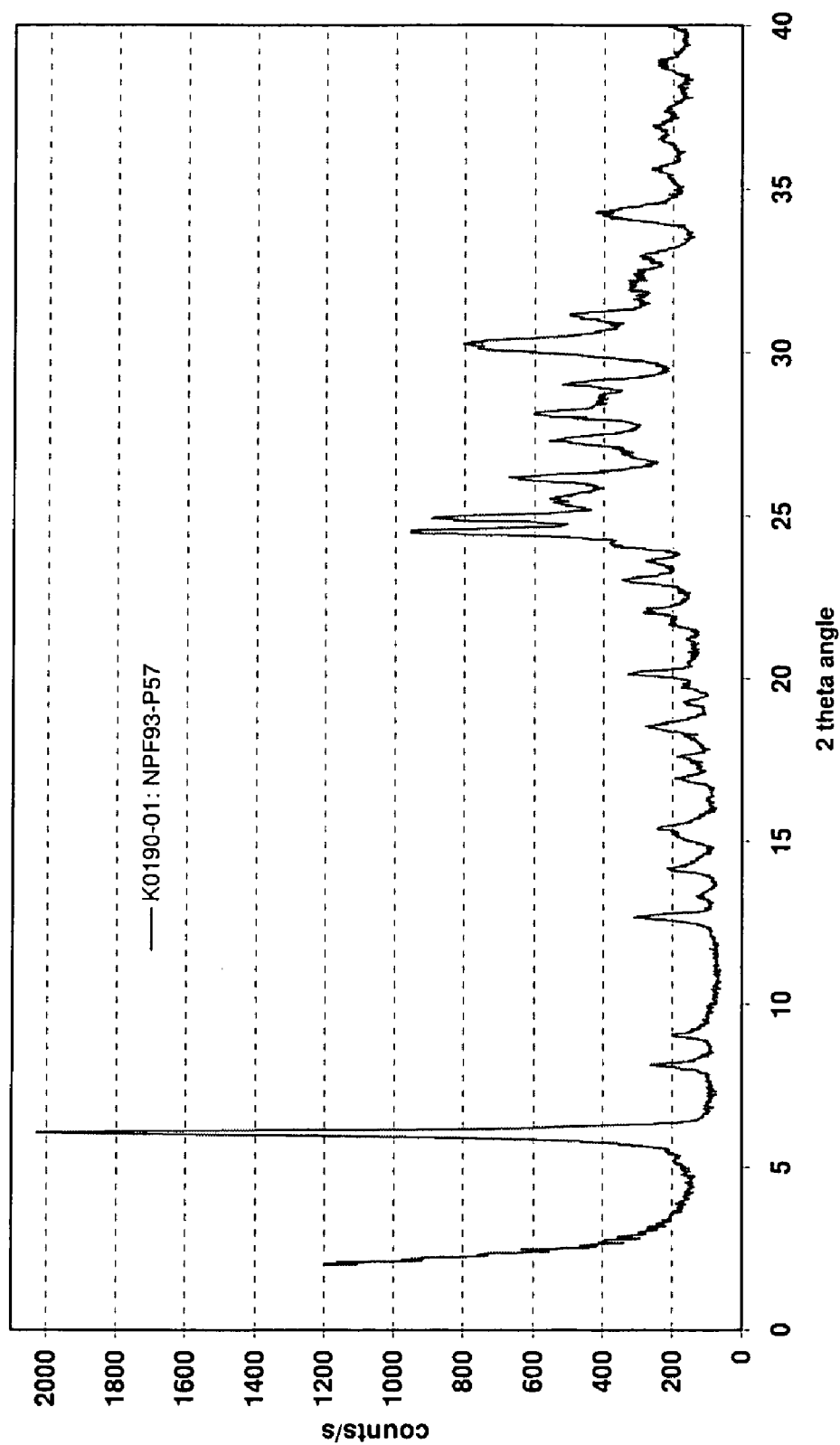
**Figure 10**

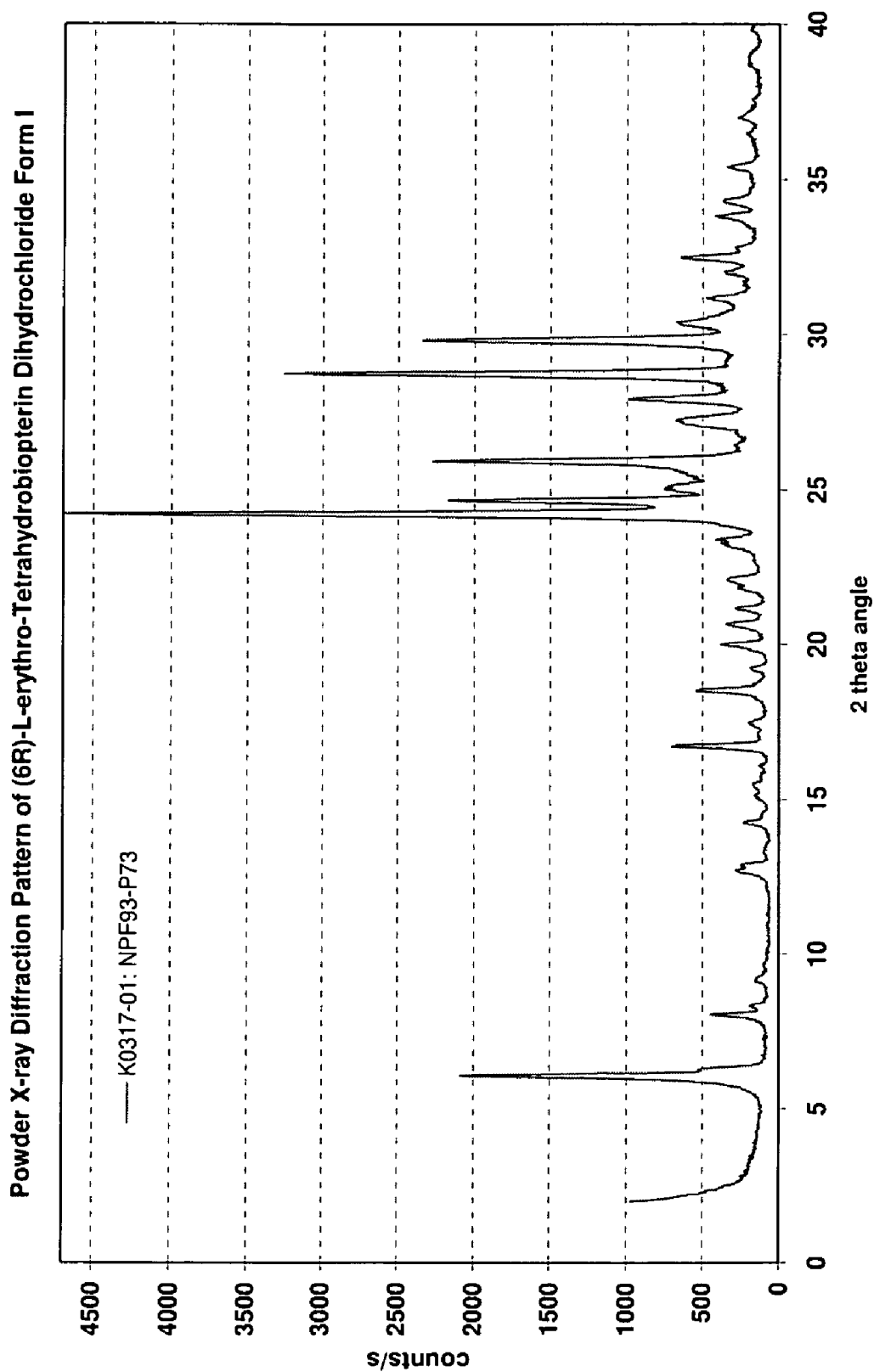
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**Figure 11****Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form G**

**Figure 12**

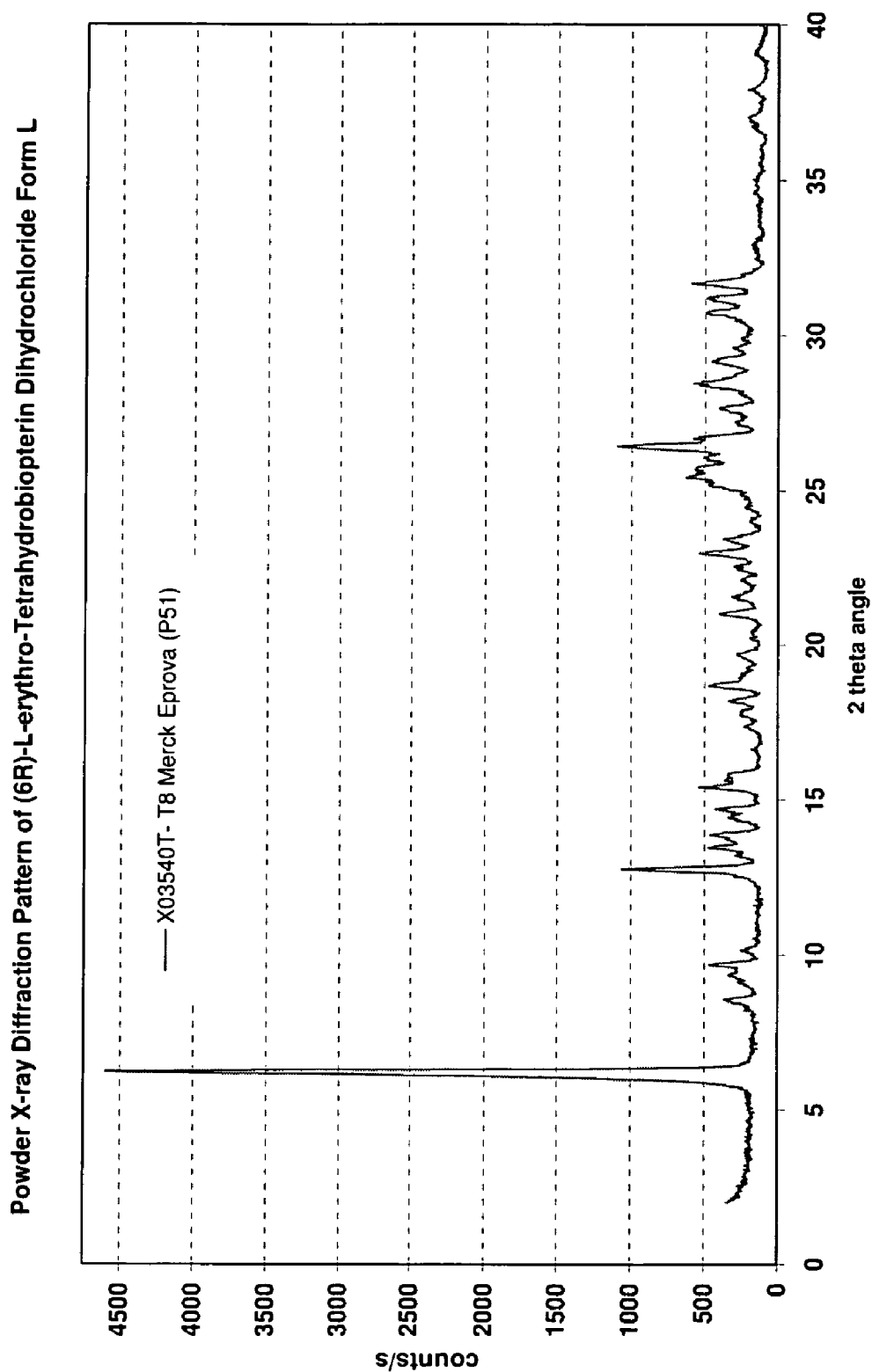
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Figure 13

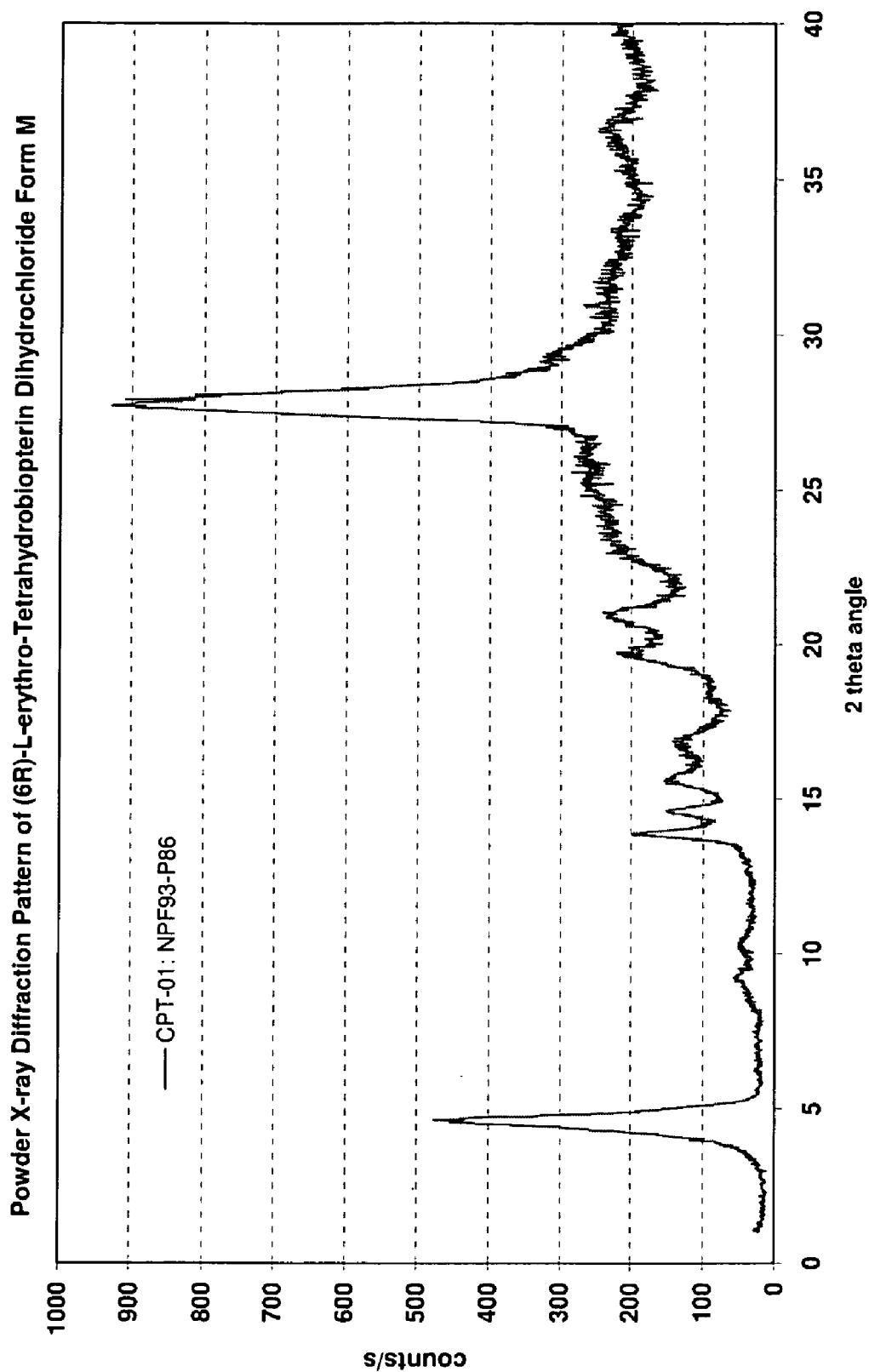


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**Figure 14**

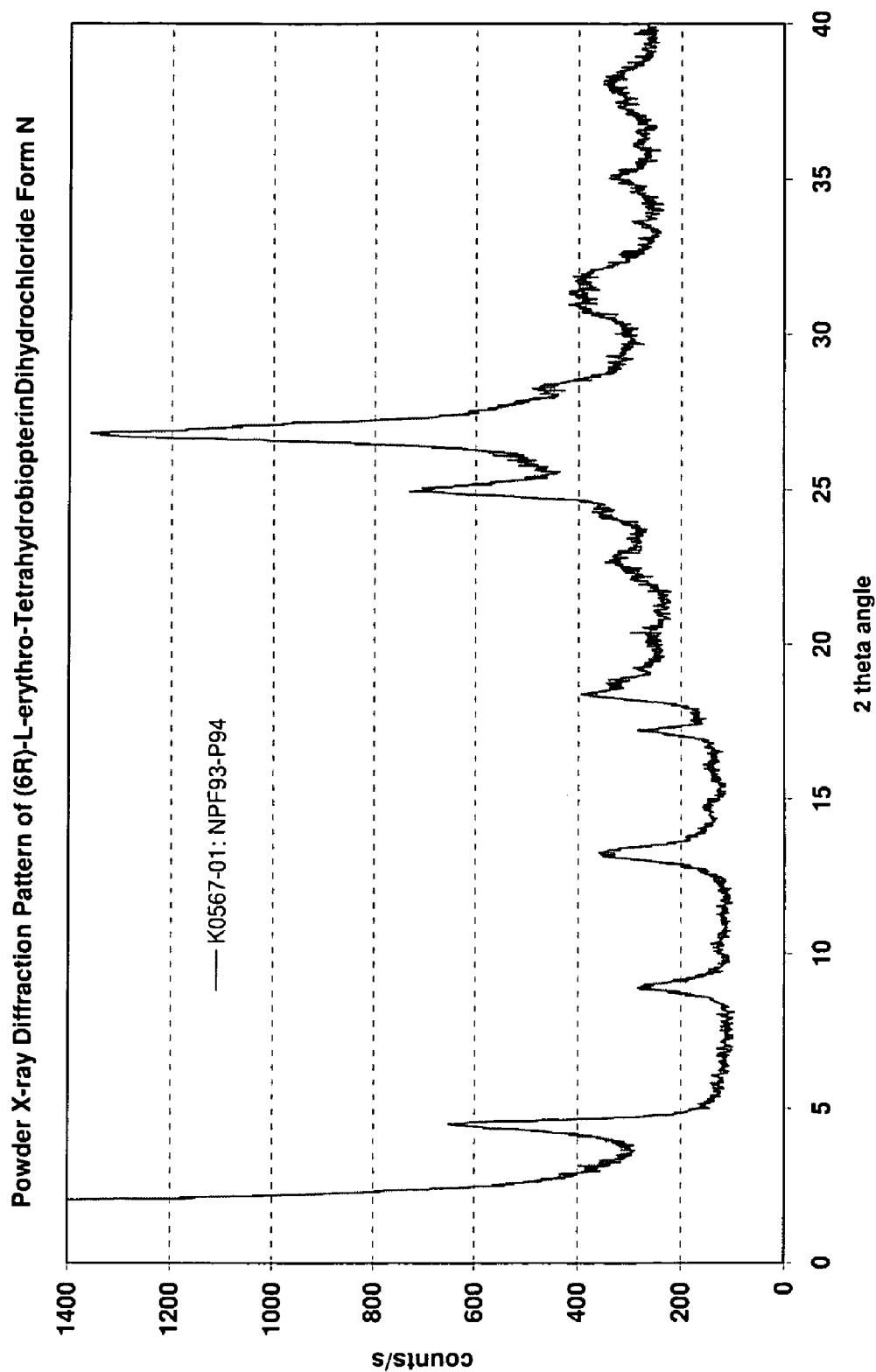
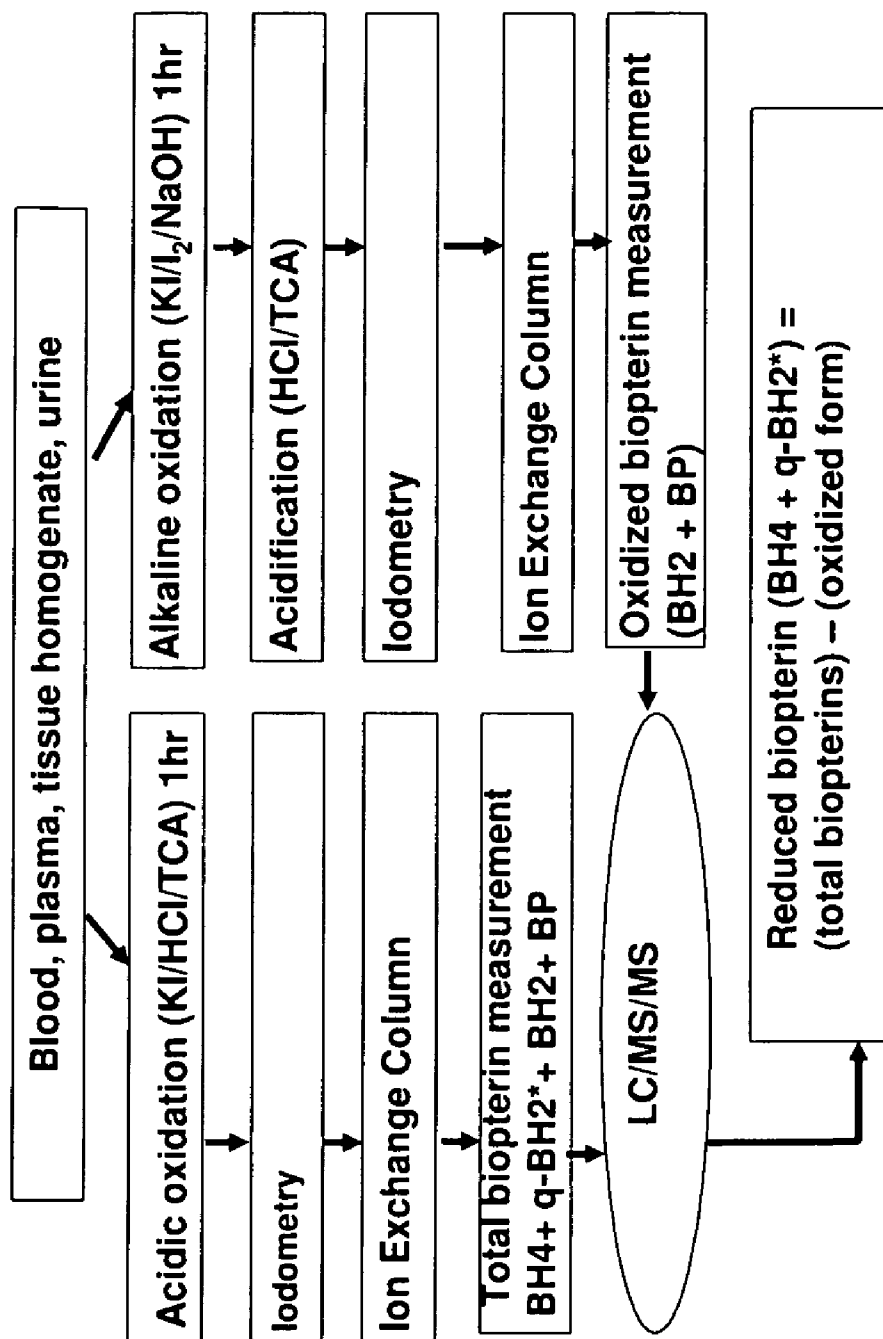
**Figure 15**



FIGURE 16 - Flow chart of biopterin measurement



\*q-BH<sub>2</sub> is immediately reduced *in vivo* to BH<sub>4</sub> so the measured reduced biopterin is based mainly upon BH<sub>4</sub>.

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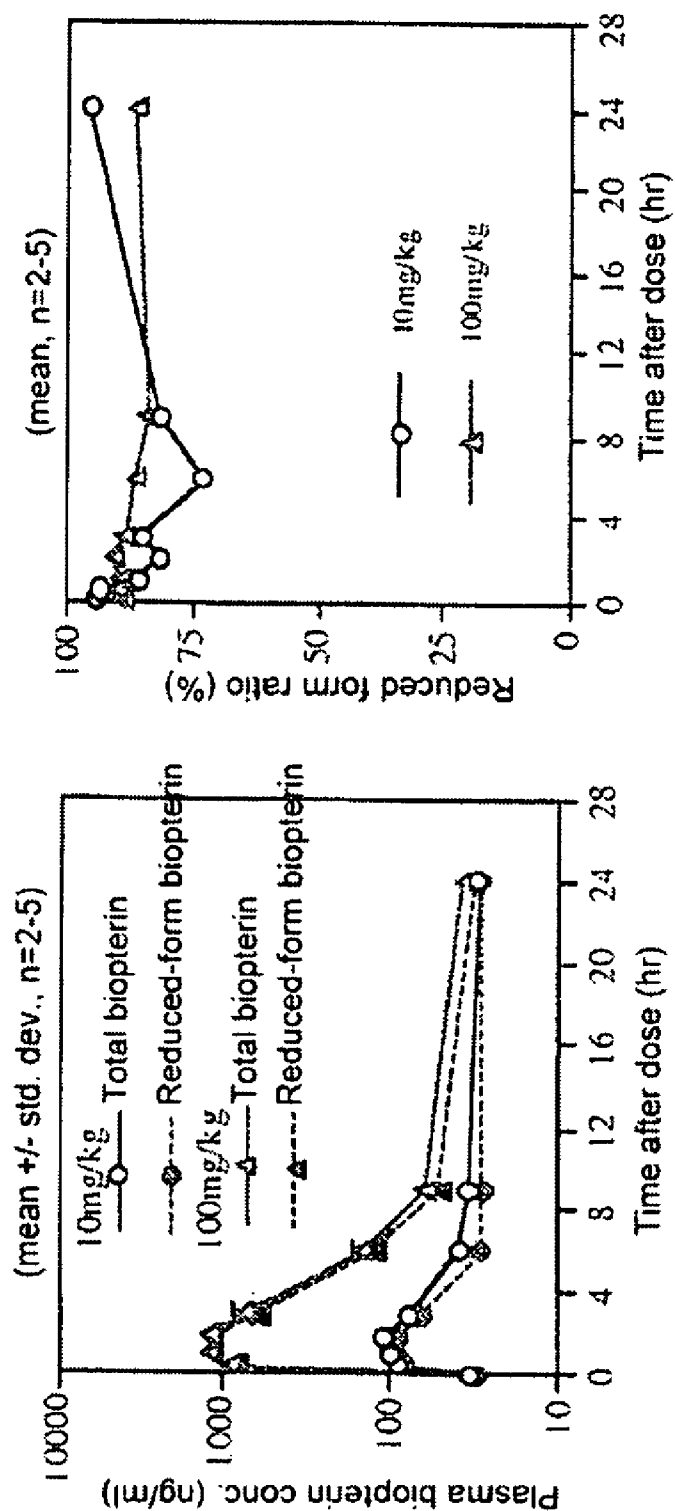
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Figure 17

Assay	LC/MS/MS Determination of BH4 in human plasma	HPLC Determination of bioppterin in human plasma
Analyte Name	Tetrahydrobiopterin (BH4)	Bioppterin
Analyte Name (oxidation product)	L-biopterin	Bioppterin
Internal Standard	Iribesartan	Bioppterin
Analytical Method Type	LC/MS/MS	HPLC (Ex 365 nm/Em 473 nm)
Extraction Method	Protein Precipitation	Protein Precipitation
QC Concentrations	5, 15, 150, and 800 ng/mL BH4	1, 10, and 40 ng/mL bioppterin
Standard Curve Concentrations	5, 15, 50, 100, 300, 500, and 1000 ng/mL	0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL
Lower Limit of Quantitation	5 ng/mL	(5 ng/mL bioppterin)
Upper Limit of Quantitation	1000 ng/mL	(50 ng/mL bioppterin)
Average Recovery of Drug	65.3%	??
Average Recovery of Internal Standard	94%	74-94%
QC Intraday Precision Range	4.7 to 14.5 %CV	0.8 to 13
QC Intraday Accuracy Range	-7.1 to 7.4 %Diff	-2.8 to 6.1
QC Interday Precision Range	7.4 to 16.4 %CV	0.6 to 4.9
QC Interday Accuracy Range	-8.3 to 3.7 %Diff	??
Stock Solution Solvent	MeOH:DMSO/50:50 (v/v)	Ammonium phosphate buffer
Benchtop Stability in human Plasma	4.5 hrs at RT	??
Freeze/thaw Stability in human plasma	4 cycles at -70 °C	2 cycles at -20 °C
Conversion Ratio from BH4 to L-Biopterin	47 % (at 12 weeks)	??
Long-term stability in K2 EDTA plasma	38 days at -70 °C	?? (7 days at -20 °C)
Dilution Integrity	1500 ng/mL diluted 10-times	??
Selectivity	BH4	Total bioppterin

FIGURE 18

Plasma Biopterin Concentration And Reduced-Form Ratio  
After Single-Dose Administration Of Sapropterin to Rats



**Figure 19**  
**Pharmacokinetic Parameters of Total Biopterins in Plasma**  
**after a Single Oral Administration of Sapropterin to Rats**

Dose (mg/kg)	Administration Route	Cendo <sup>1</sup> (ng/ml)	Cmax (ng/ml)	$\Delta C_{max}^{2^2}$ (ng/ml)	Tmax (hr)	$\Delta AUC^3$ (ng·hr/ml)	T <sub>1/2</sub> (h)	F <sup>4</sup> (%)
100	p.o.	38.2	1227	1189	1.0	4571	1.1(2-6 h)	11.8
10	p.o.	33.5	108	75	2.0	265	1.1(2-6h)	6.8
10	i.v.	33.5	—	—	—	3881	0.6(0.5–3h)	
1	i.v.	33.5	—	—	—	529	0.3(0.5–3h)	

<sup>1</sup> Endogenous total bioppterin concentration

<sup>2</sup> Cmax – Cendo

<sup>3</sup> Computed based on trapezoidal rule, by using the value ( $\Delta C$ ) obtained by subtracting Cendo from the actually measured value (C) of plasma concentration.

<sup>4</sup> Bioavailability (F) was computed by using  $\Delta AUC$  at the time of 10-mg/kg intravenous injection:

$$F = [\Delta AUC_{po}] / [DOSE_{po}] / [\Delta AUC_{iv}] / 10 \times 100 (\%)$$

Sapropterin Lot #s: 8886202, 8885Y05

(mean values of 2 to 5 animals)

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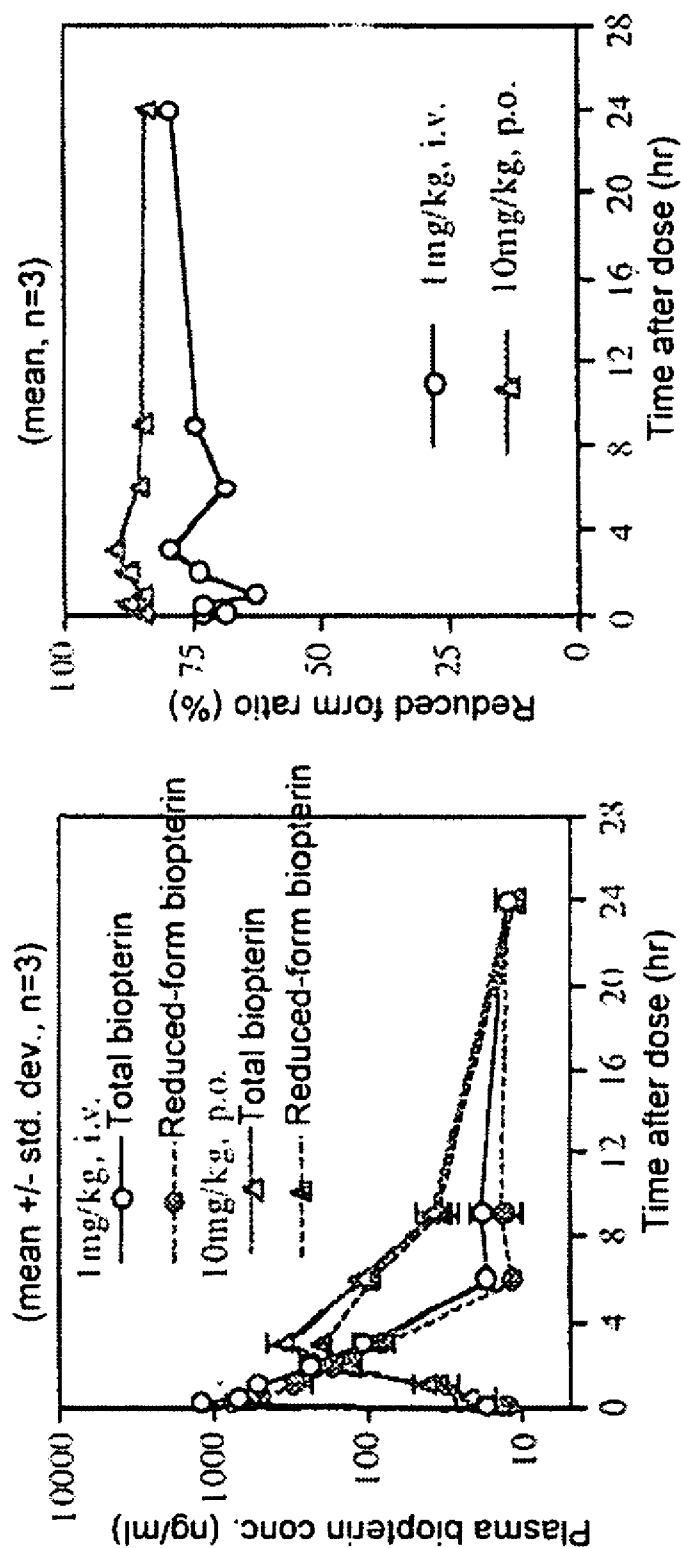
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FIGURE 20

# **Plasma Biopterin Concentration and Reduced-Form Ratio After a Single-Dose Administration of Sapropterin in Monkeys**



**FIGURE 21**

**Pharmacodynamic Parameters of Total Biopterins in Plasma  
after Single-Dose Administration of Saproterin to Monkeys**

Dose (mg/kg)	Administration Route	Cendo <sup>1</sup> (ng/ml)	Cmax (ng/ml)	$\Delta$ Cmax <sup>2</sup> (ng/ml)	Tmax (hr)	$\Delta$ AUC <sup>3</sup> (ng-hr/ml)	T <sub>1/2</sub> (hr)	F <sup>4</sup> (%)
10	p.o.	17.4±1.3	344±149	344±148.5	2.9±0.2	1301±144	1.42±0.17	9.0
1	i.v.	17.1±2.1				1449±68.4	0.82±0.14	

<sup>1</sup> Endogenous total biopterin concentration

<sup>2</sup> Cmax – Cendo

<sup>3</sup> Computed based on trapezoid rule, by using the value ( $\Delta$ C) obtained by subtracting Cendo from the actually measured value (C) of plasma concentration.

<sup>4</sup> Bioavailability (F) was computed by using  $\Delta$ AUC at the time of 1-mg/kg intravenous injection.

$F = [\Delta\text{AUC}_{\text{Cpo}}] / [\text{DOSE}_{\text{Epo}}] / [\Delta\text{AUC}_{\text{Civ}}] / 1 \times 100 (\%)$

Saproterin Lot #s: 8886202, 8885Y05

(mean value ± standard error, n = 3)



Figure 22: Schedule of Events

Visit	Open-Label Treatment					Follow-up
	Screening	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5
Informed consent	X					
Weight	X	X <sup>5</sup>				X
Vital signs	X	X	X		X	X
Physical examination	X					X
Clinical laboratory tests <sup>1</sup>	X					X
Pregnancy test <sup>4</sup>	X	X			X	X
Urine Drugs of Abuse Screen <sup>2</sup>	X	X	X	X	X	X
Concomitant medications	X	X	X	X	X	X
Adverse events		X	X	X	X	X
Blood PK sampling <sup>3</sup>		X	X	X	X	X
Dispense study drug		X	X	X	X	

<sup>1</sup> Clinical laboratory tests included hematology, chemistry, urinalysis, Hepatitis B & C, and HIV at screening. Hematology, chemistry and urinalysis only were repeated at study discharge. Approximately 20 mL of blood was collected at visits that included clinical laboratory tests.

<sup>2</sup> Urine drugs screen to include testing for amphetamines, benzodiazepines, barbiturates, cocaine, cannabinoids, and opiates.

<sup>3</sup> Pharmacokinetic samples were taken at the following timepoints during each treatment period: pre-dose, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post dose. Approximately 80 mL of blood was drawn during each treatment period (5 mL per timepoint) for the PK analysis.

<sup>4</sup> Additional urine pregnancy tests were performed at any visit at which pregnancy status was in question, and serum pregnancy tests were performed in the event of any positive or equivocal urine pregnancy test results.

<sup>5</sup> Weight at the Week 1 treatment period was used to calculate dose.

FIGURE 23

**Mean Plasma Concentrations of BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers – Linear Axes**

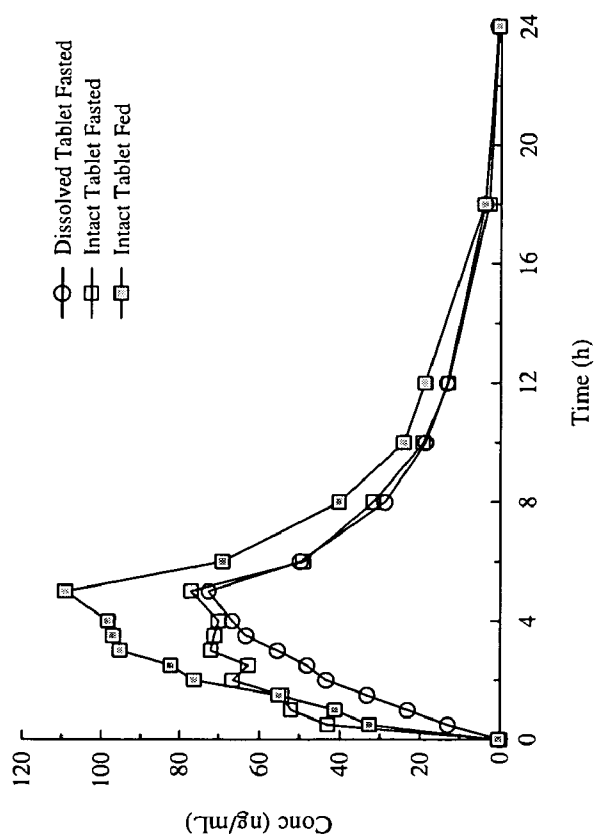


FIGURE 24

Mean Plasma Concentrations of  $\text{BH}_4$  after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers – Semi-Logarithmic Axes

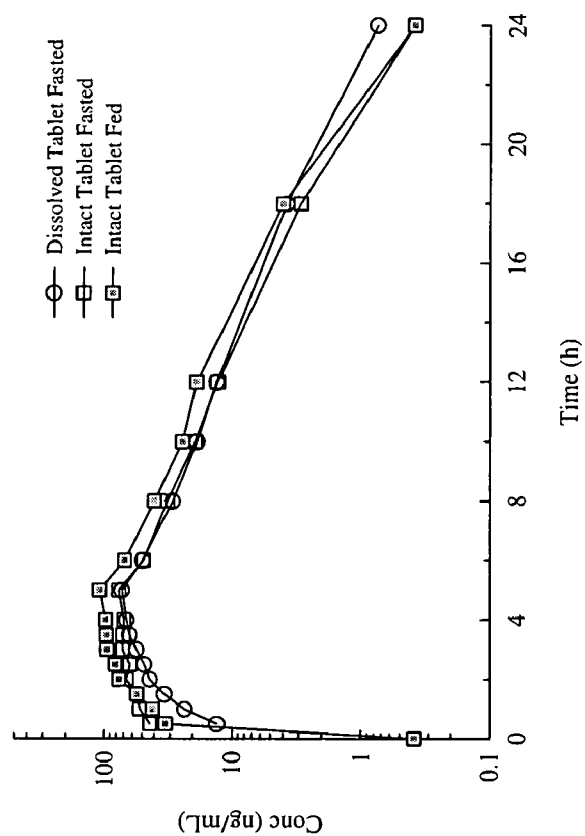


FIGURE 25

**Summary of Pharmacokinetic Parameters for BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers**

Parameter <sup>1</sup>	Dissolved Tablet Fasted	Intact Tablet Fasted	Intact Tablet Fed
C <sub>max</sub> (ng/mL)	80.3 ± 63.3 [30] (69.4)	91.2 ± 36.3 [30] (84.0)	121 ± 33.6 [30] (116)
T <sub>max</sub> (h)	4.00 [30] (2 - 6)	3.50 [30] (1 - 5)	4.00 [30] (1 - 5)
AUC(0-t) (h•ng/mL)	479 ± 292 [30] (420)	550 ± 214 [30] (505)	709 ± 221 [30] (675)
AUC(inf) (h•ng/mL)	597 ± 336 [22] (528)	704 ± 202 [19] (670)	825 ± 256 [23] (784)
λ <sub>z</sub> (h <sup>-1</sup> )	0.2101 ± 0.1326 [22]	0.2099 ± 0.0942 [19]	0.2104 ± 0.0918 [23]
t <sub>1/2</sub> (h)	5.31 ± 4.42 [22]	4.47 ± 3.37 [19]	4.28 ± 2.79 [23]

<sup>1</sup>Mean ± standard deviation except for T<sub>max</sub> for which the median is reported. Numbers in square brackets are the number of subjects for which the parameter could be estimated and numbers in parentheses are the geometric means for C<sub>max</sub>, AUC(0-t), and AUC(inf) and the ranges for T<sub>max</sub>.

FIGURE 26

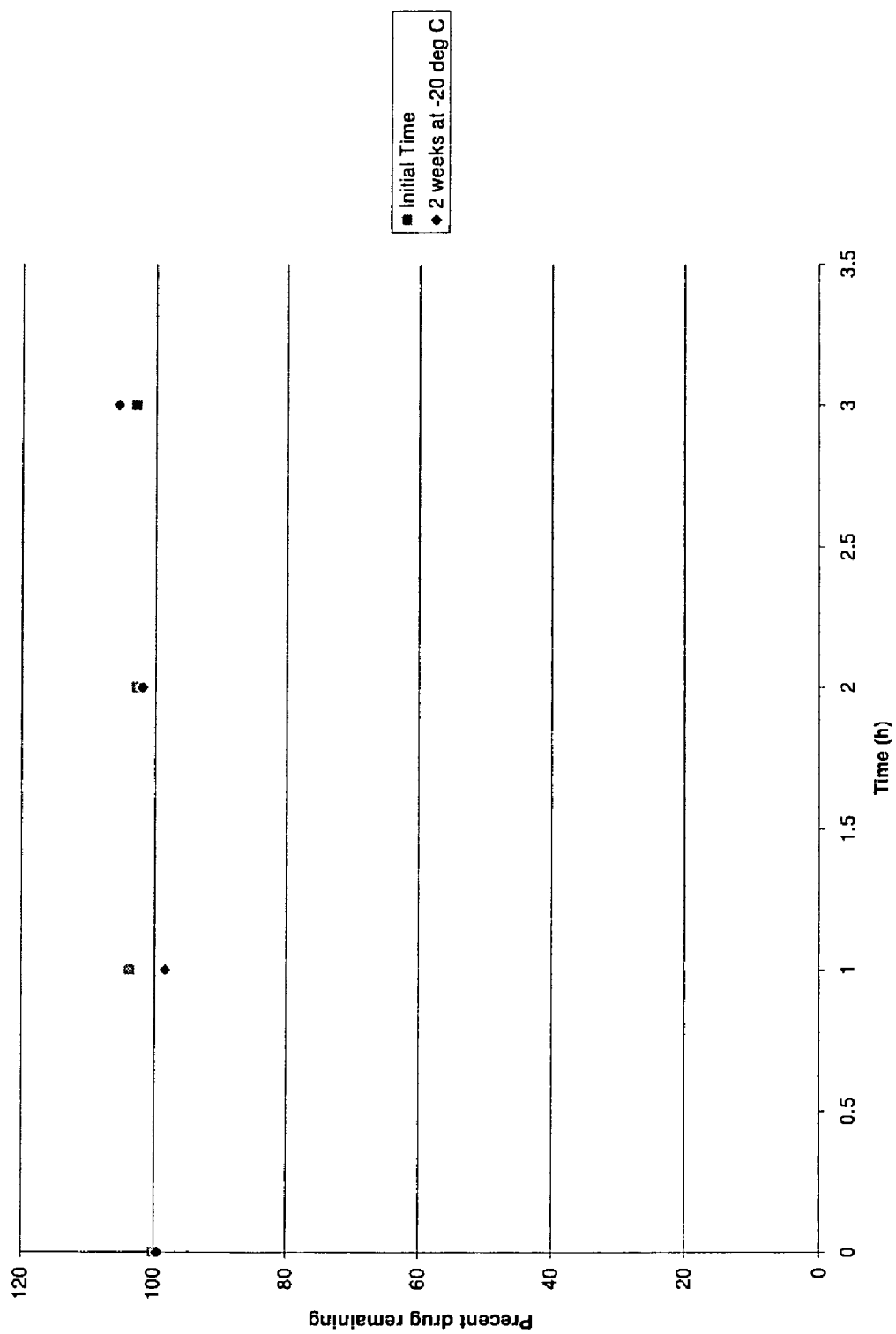
Statistical Comparison of Pharmacokinetic Parameters for BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers

Parameter	Geometric Mean Ratio (%) <sup>1</sup>	
	Estimate	90% Confidence Interval
Intact vs. Dissolved Tablet (Fasted)		
C <sub>max</sub>	120.98	104.21 → 140.44
AUC(0-t)	120.33	104.12 → 139.06
AUC(inf)	118.04	98.16 → 141.96
Intact Tablet — Fed vs. Fasted		
C <sub>max</sub>	138.63	119.42 → 160.93
AUC(0-t)	133.69	115.68 → 154.50
AUC(inf)	125.61	104.29 → 151.30

<sup>1</sup>Based on analysis of natural log-transformed data.

FIGURE 27

Stability of BH4 (1 mg/mL) in 5% mannitol aqueous solution



**FIGURE 28**  
Dissolution Profile GMO Capsule Prototype

USP Paddle Apparatus, 50 rpm, 37°C, 900 mL 0.1 N HCl (110210-28, 110210-76E)

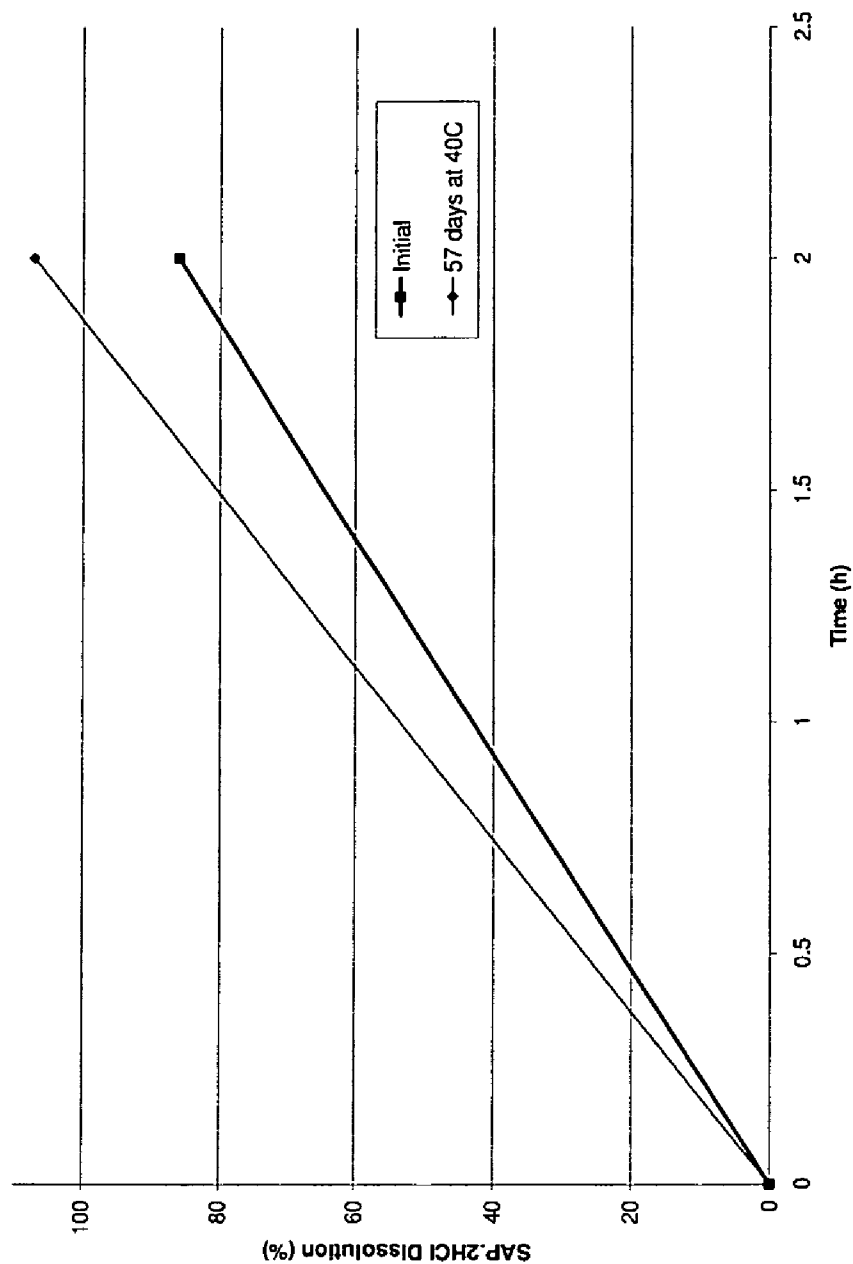
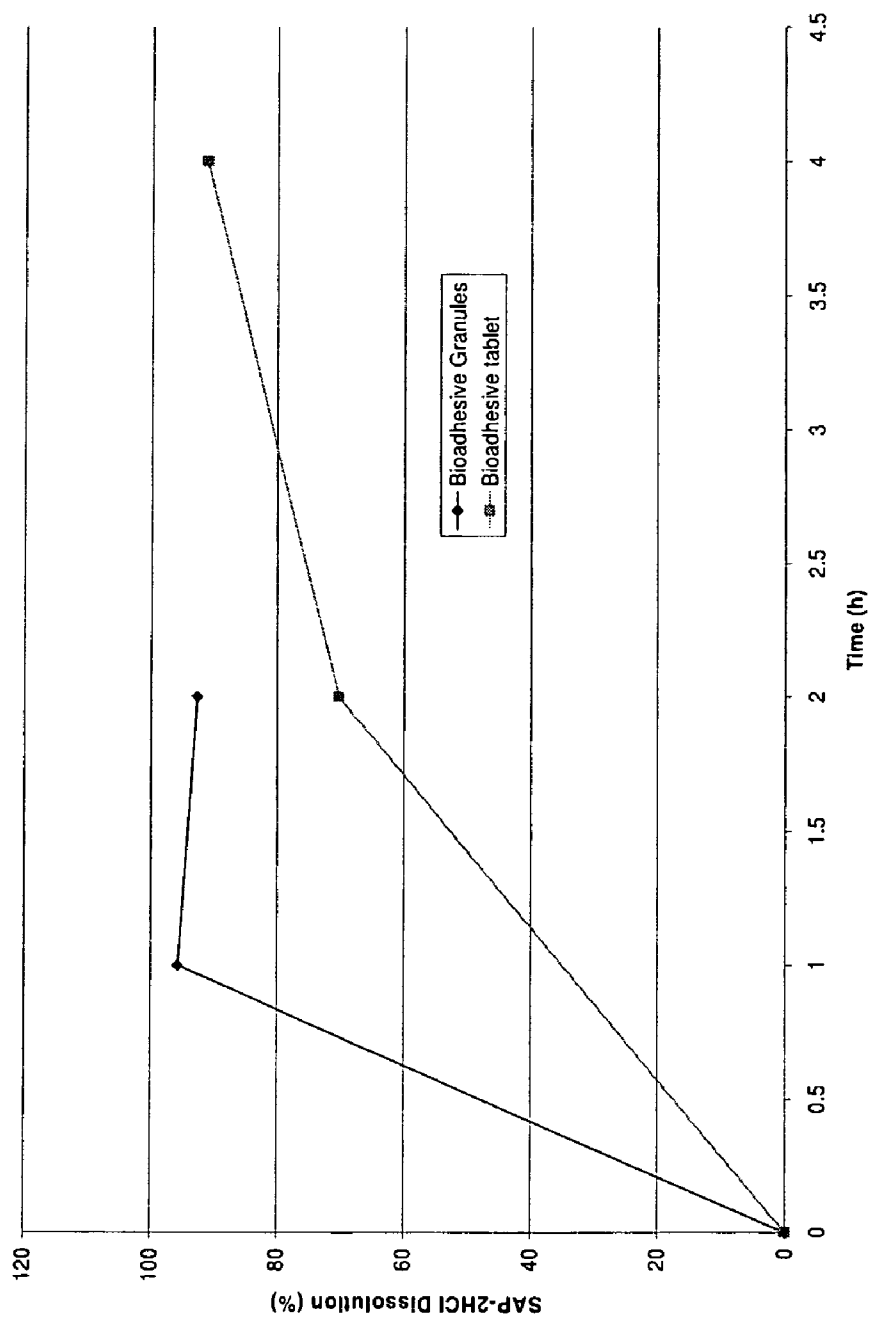




FIGURE 29

Dissolution Profile of Bioadhesive Tablet Prototype (80 mg BH4)

USP Paddle Apparatus, 50 rpm 37°C, 900 mL 0.1N HCl (11229-04, 11229-85)



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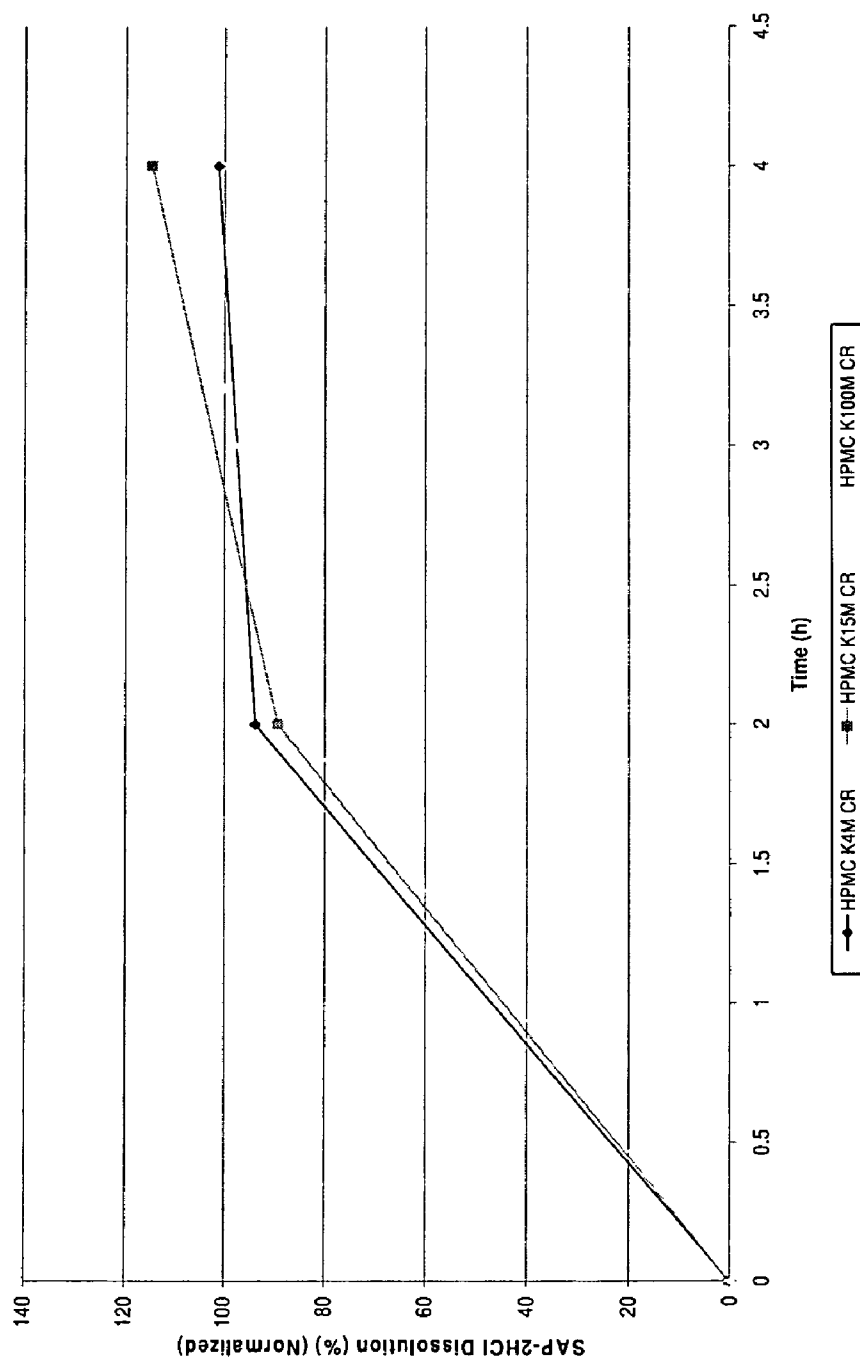
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FIGURE 30

Dissolution Profile of Sustained Release Prototype (80 mg SAP-2HCl) with 20% HPMC of various viscosity grade

USP Paddle Apparatus, 50 rpm, 37°C, 900 mL 0.1N HCl



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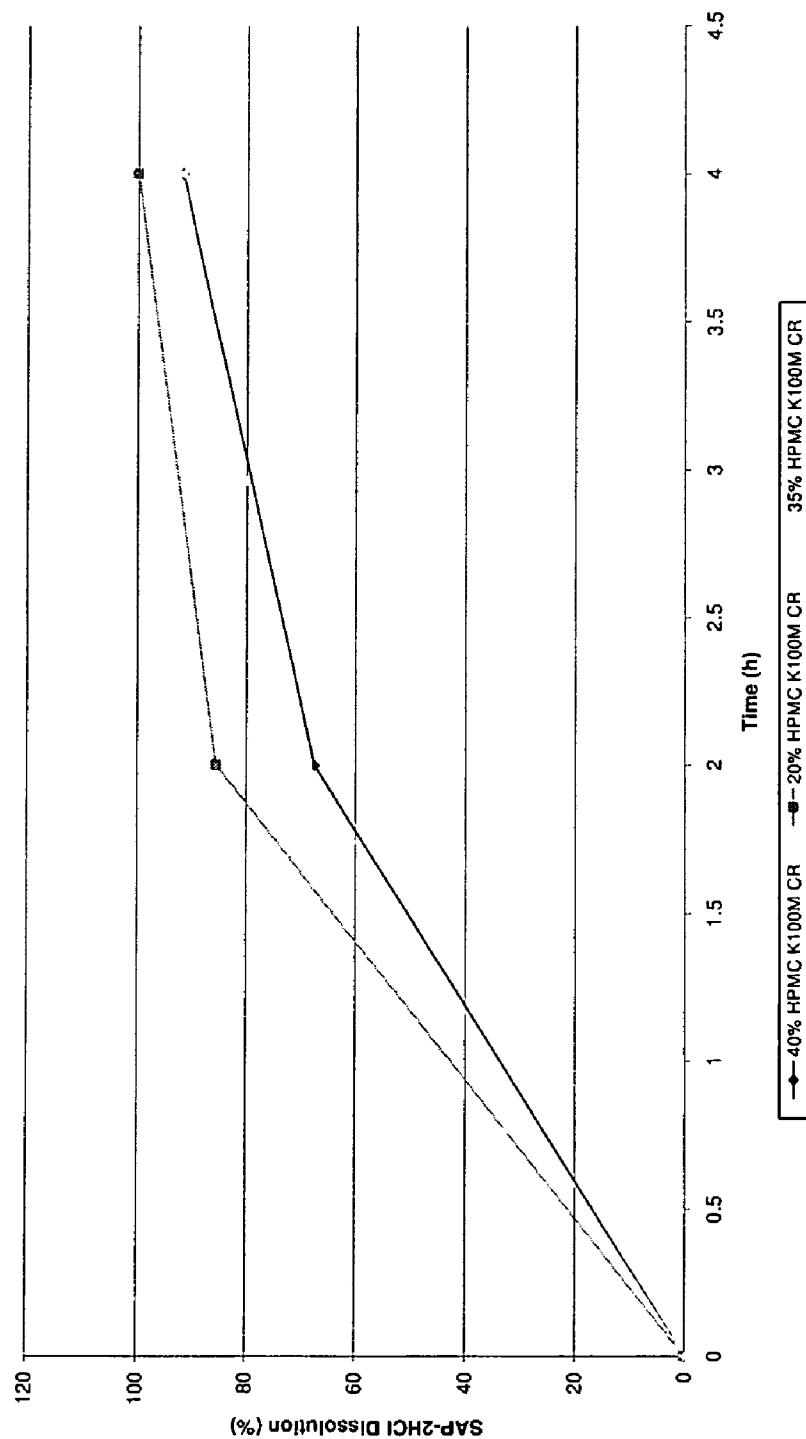
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FIGURE 31

Dissolution Profile of Sustained Release Prototypes (80 mg BH4) containing 20% to 40% Methocel K100M CR

USP Paddle Apparatus, 50 rpm, 37°C, 900 mL 0.1N HCl



## FIGURE 32

### Schematic Diagram of Floating Dosage Form

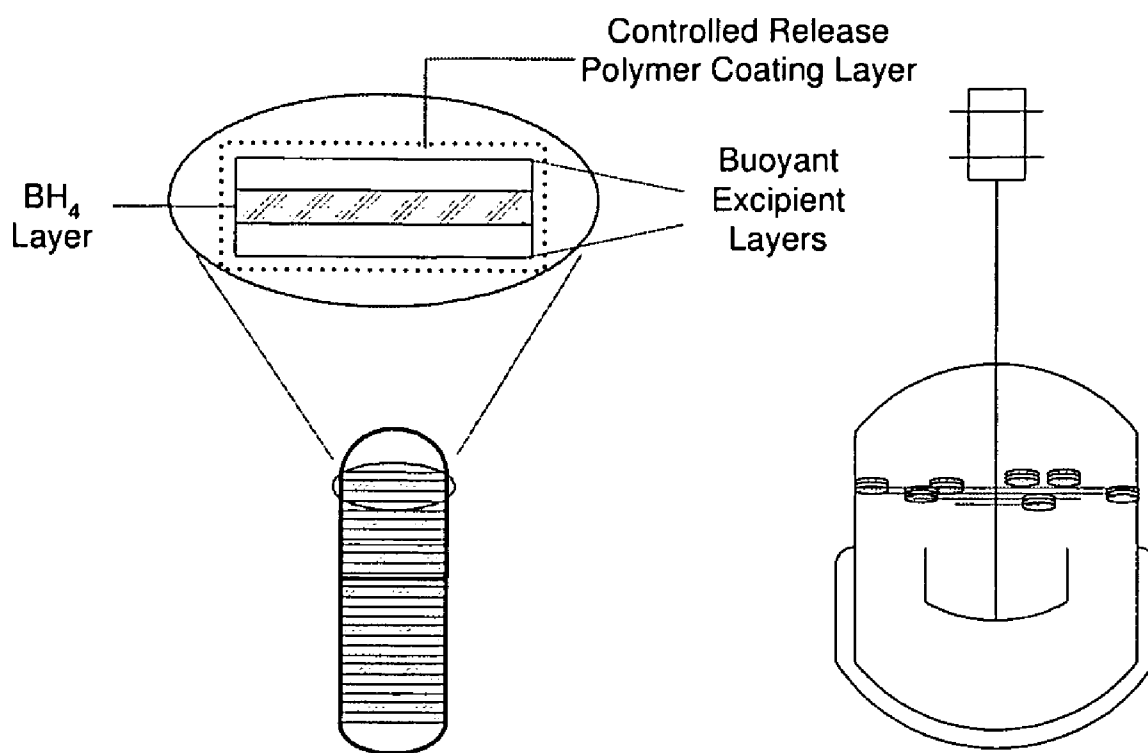


FIGURE 33

Dissolution Profile of Floating System Prototype (80 mg BH4) with Varying Levels of PEG Coating

USP Paddle Apparatus, 50 rpm 37 °C, 900 mL 0.1N HCl

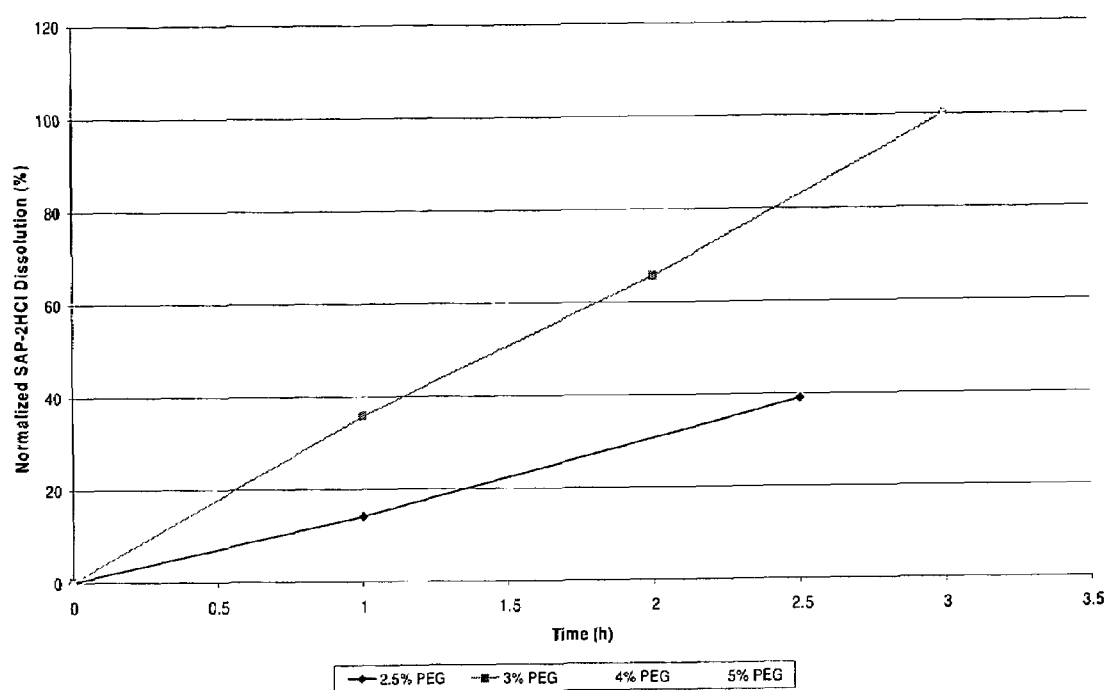


FIGURE 34

Schematic Diagram of Gas Generating Dosage Form

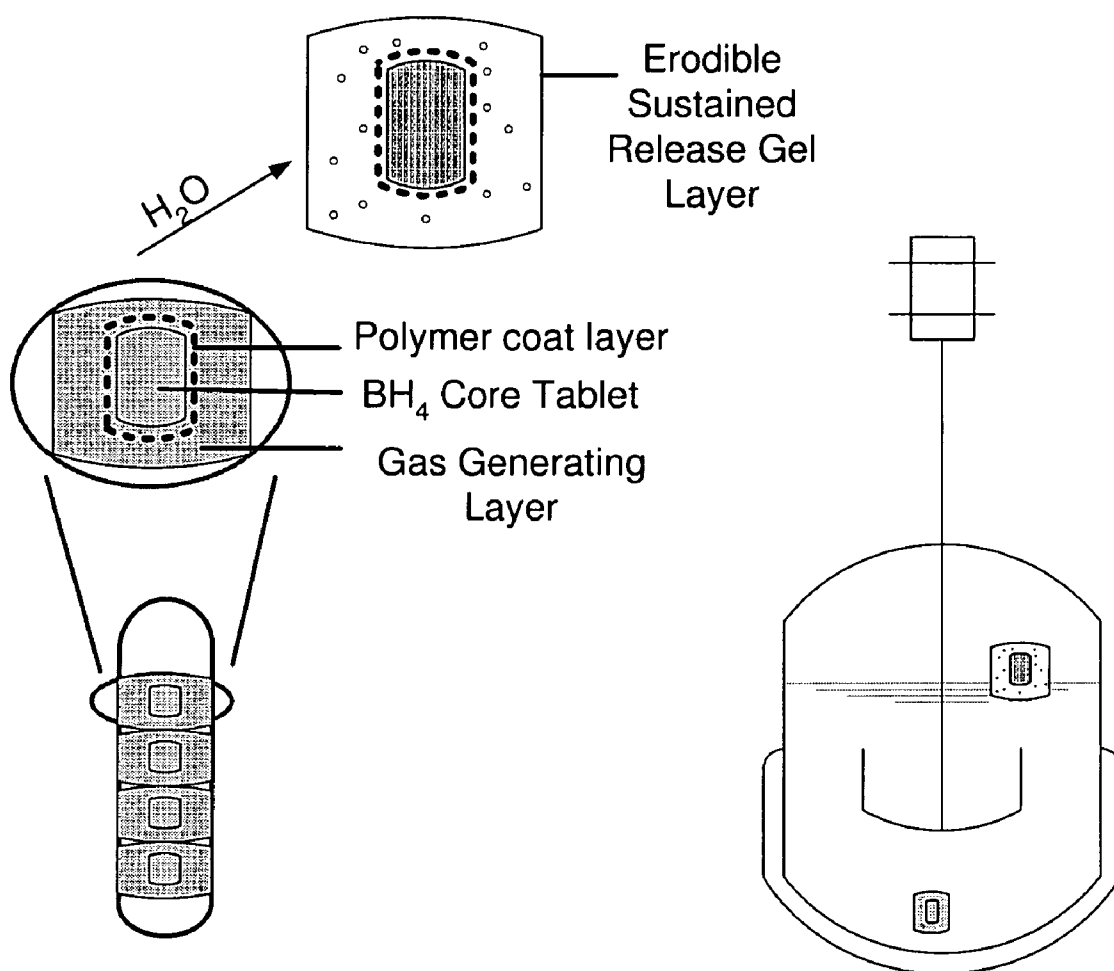


FIGURE 35

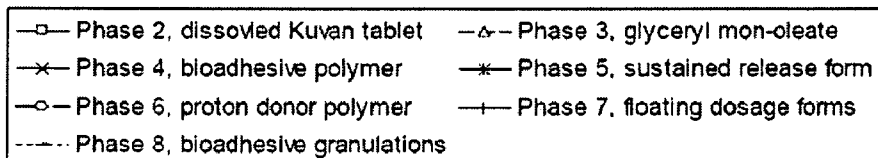
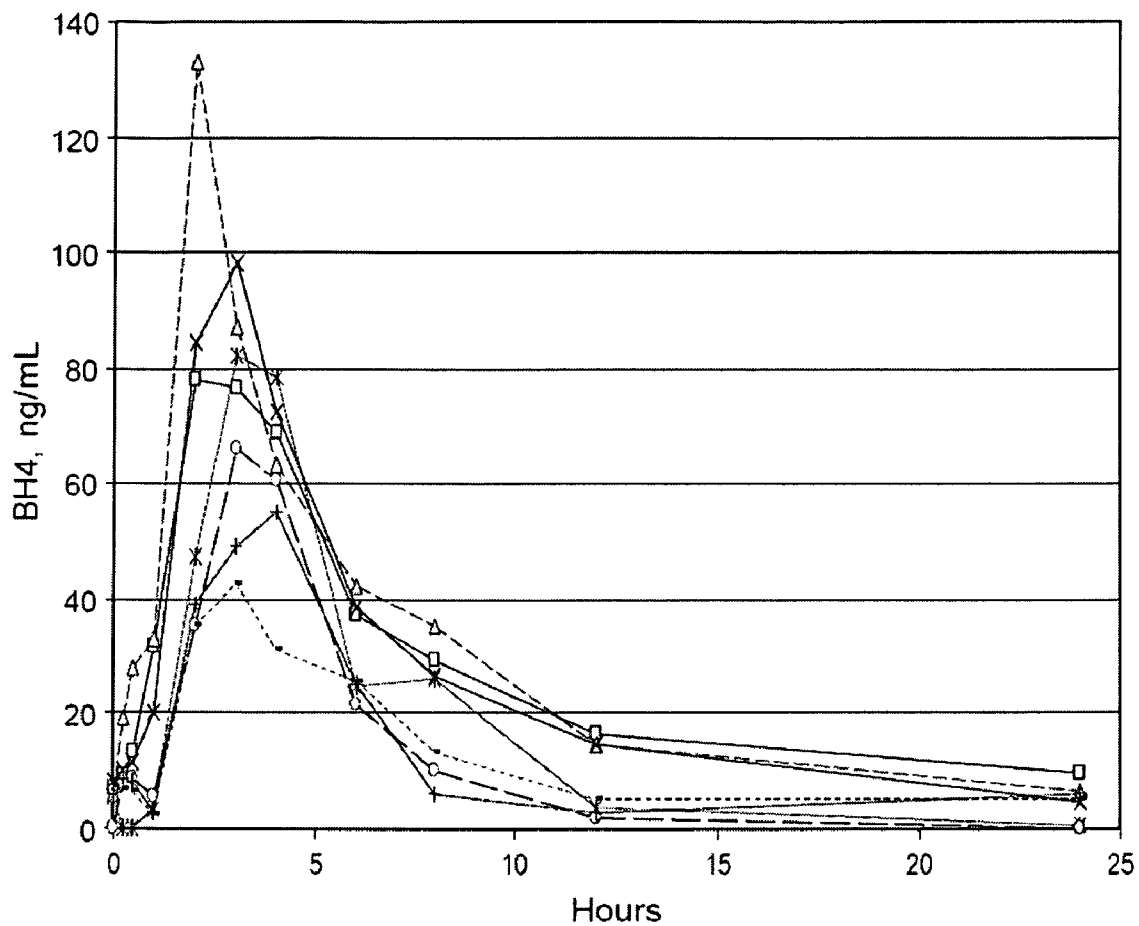




FIGURE 36

Stability of BH4 in pH 4 Buffer in the Presence and Absence of Antioxidants and Sparging with Argon

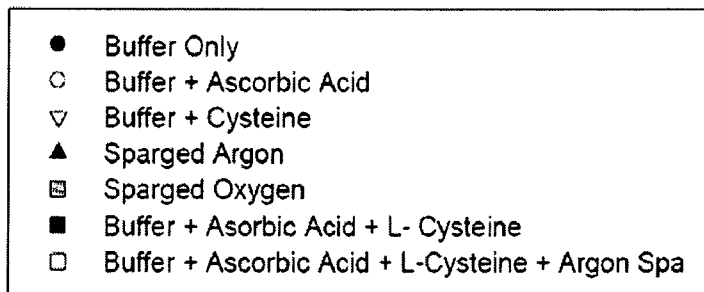
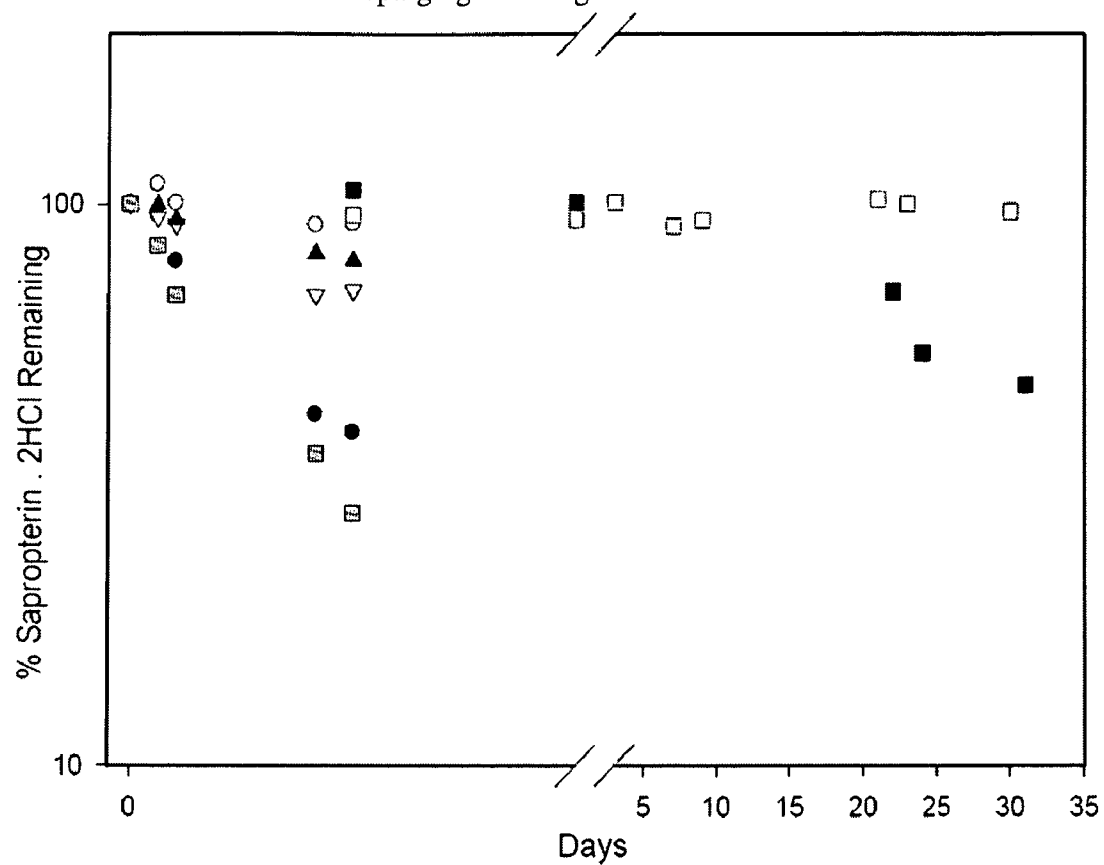


FIGURE 37

Stability of BH4 in pH 7 Buffer in the Presence and Absence of Antioxidants and Sparging with Argon

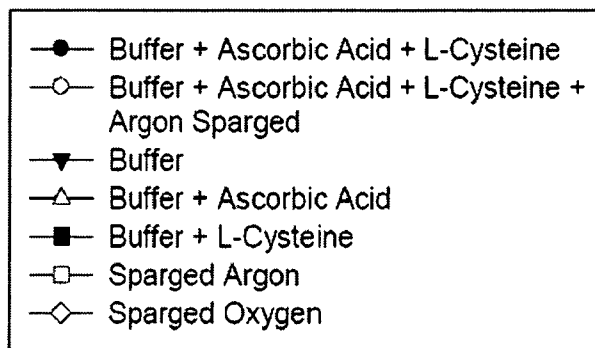
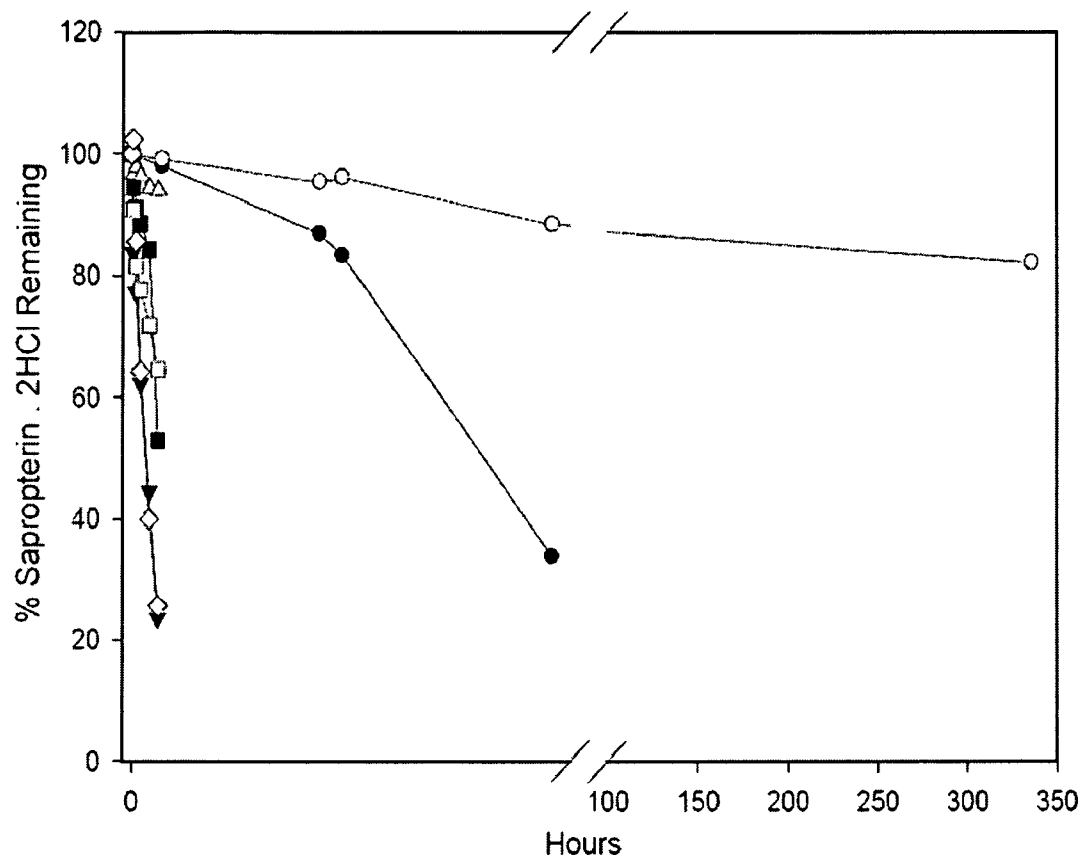
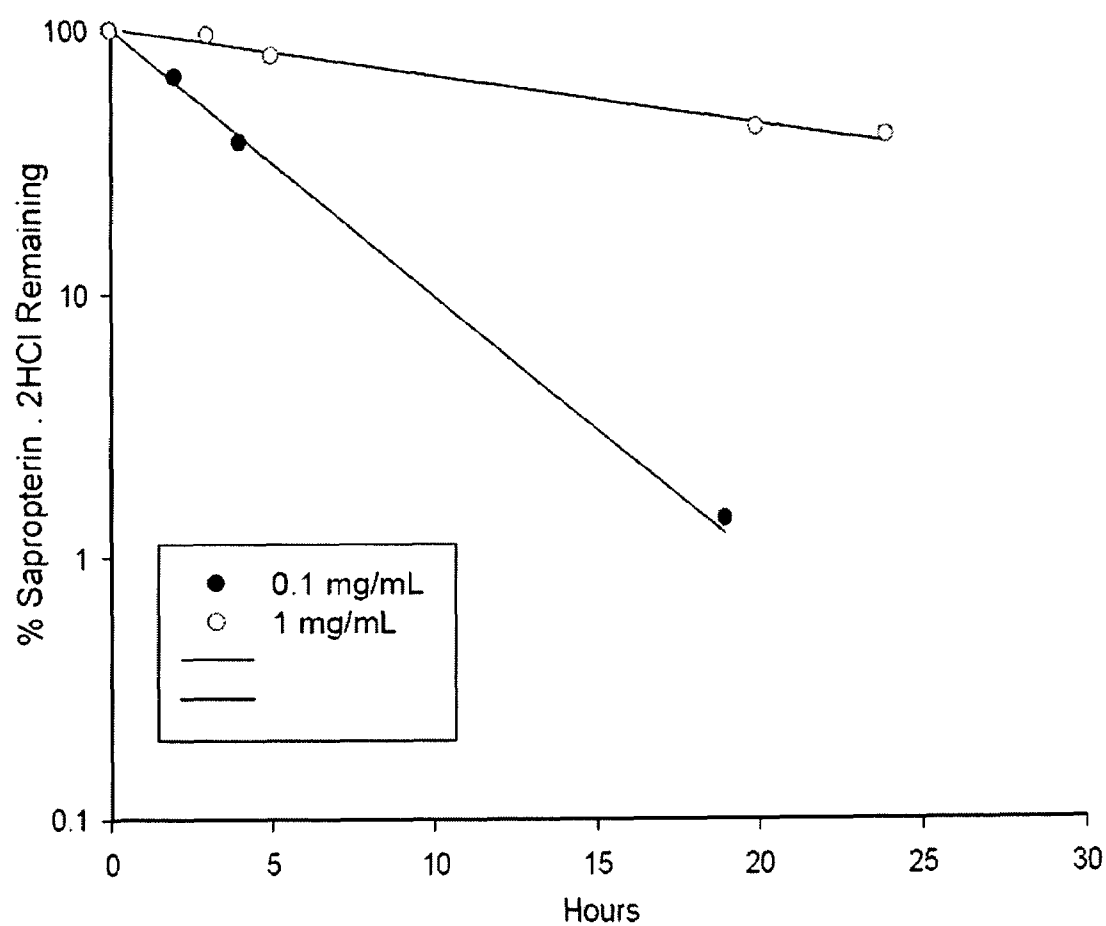


FIGURE 38

Stability of BH4 Remaining in pH 4 Buffer Solution with Time as a Function of Concentration



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# METHODS OF ADMINISTERING TETRAHYDROBIOPTERIN, ASSOCIATED COMPOSITIONS, AND METHODS OF MEASURING

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of International Application No. PCT/US08/060,041, filed Apr. 11, 2008, which claims priority to U.S. Provisional Application Nos. 60/922,821, filed Apr. 11, 2007, and 61/019,753, filed Jan. 8, 2008, the disclosures of which are incorporated herein by reference in their entirety.

## BACKGROUND

### 1. Field

The present invention is generally directed to compositions and methods for treating BH4-responsive disorders, and methods and compositions for detecting and quantitating bipterins.

### 2. Background of the Related Technology

Tetrahydrobiopterin (referred to herein as BH4) is a biogenic amine of the naturally-occurring pterin family that is a cofactor for a number of different enzymes, including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. It is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (*Disorders of tetrahydrobiopterin and related biogenic amines*. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1275-1776).

Feige, et al., *Molecular Genetics and Metabolism* 81:45-51 (2004) studied pharmacokinetics of orally administered tetrahydrobiopterin (BH4) and suggested a "rather large variability of orally administered BH4, probably due to different absorption in the gut and/or to the first passage effect."

Use of tetrahydrobiopterin has been proposed for treating a variety of different disease states, and there exists a need for alternative and improved methods of administering this drug.

## SUMMARY OF THE INVENTION

The present invention relates to methods of administering 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4), or a pharmaceutically acceptable salt thereof, in a manner that improves or maximizes its oral bioavailability and/or improves or optimizes the consistency of oral bioavailability from one administration to the next. Such methods can be applied in the treatment of any BH4-responsive disorder, including metabolic diseases, cardiovascular diseases, anemia, and neuropsychiatric disorders. The methods of the invention advantageously allow better control of clinical symptoms, e.g. reduced fluctuation in plasma phenylalanine levels, blood pressure, neurotransmitter levels, or other clinical parameters.

As used herein, BH4 refers to 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin. The term BH4 as used herein is also to be understood to optionally mean a pharmaceutically acceptable salt of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin, unless the context dictates otherwise.

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In a first aspect, the invention provides methods of orally administering to a patient in need thereof a purified preparation of BH4.

In an exemplary embodiment, the methods comprise the step of informing the patient that absorption of tetrahydrobiopterin is increased when it is ingested with food compared to when ingested without food. In some embodiments, the patient is informed that ingestion shortly following a meal, for example, a high-fat, high-calorie meal, results in an increase in any one, two, three or all of the following parameters: mean plasma concentration, Cmax, AUC, AUC(0-t) and/or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 with a high-fat meal increases Cmax and AUC compared to administration of BH4 without food (in a fasting condition). In some embodiments, the relative increase can be at least 20% or 30% or more.

In alternative embodiments or in addition to the preceding embodiments, the method of administering tetrahydrobiopterin comprises informing the patient that absorption of tetrahydrobiopterin is increased when ingested as an intact tablet compared to when ingested after being dissolved in liquid. In some embodiments, the patient is informed that ingestion of intact tablets results in an increase in any of the following parameters: mean plasma concentration, Cmax, AUC, AUC(0-t) or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 as an intact tablet increases Cmax and AUC compared to administration of BH4 after being dissolved in a liquid. In some embodiments, the relative increase can be at least 20% or more.

Any of the preceding methods may be carried out by providing or administering tetrahydrobiopterin in a container containing printed labeling informing the patient of the change in absorption parameters described above.

Optionally, the methods of the invention also comprise the step of providing to the patient in need thereof a therapeutically effective amount of tetrahydrobiopterin. The therapeutically effective amount will vary depending on the condition to be treated, and can be readily determined by the treating physician based on improvement in desired clinical symptoms.

In one exemplary embodiment, such methods involve administering BH4 in a dissolved form, wherein the formulation is dissolved in a liquid including but not limited to water, orange juice and apple juice. In one exemplary embodiment, dissolved BH4 is administered to the patient in a fasted condition, i.e., on an empty stomach. The invention further contemplates that the dissolved BH4, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal. Thus, BH4 may be ingested as a liquid product or pre-dissolved from a solid or semisolid dosage form prior to ingestion. In a further embodiment, BH4 may also be dissolved in the oral cavity from a solid or semisolid dosage form prior to swallowing the dissolved solution.

In another exemplary embodiment, such methods involve administering BH4 in a solid dosage form including but not limited to tablets, capsules, candies, lozenges, powders, and granules, or semisolid form, including but not limited to oral sprinkle into jelly, that is swallowed without dissolving in a liquid including but not limited to water, orange juice and apple juice, before swallowing. In one embodiment, swallowed BH4 is administered to the patient in a fasted condition,

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i.e. on an empty stomach. The invention further contemplates that the BH4 swallowed as a solid or semisolid dosage form, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal.

In another embodiment, such methods involve administering BH4, whether swallowed as a solid or semisolid dosage form, or dissolved in a liquid, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal. The invention further contemplates that BH4, whether swallowed or dissolved, is administered at a specified time including but not limited to morning, day, night, same time of the day, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal, one or more times a day. In an exemplary embodiment, BH4 is ingested once daily as a solid dosage form just after meals. In a preferred embodiment the solid dosage form is a formulated tablet or capsule. In more exemplary embodiments, BH4 is ingested within approximately 0 to 30 minutes, or 5 to 20 minutes, of eating a meal. Regardless of whether it is ingested as a solid dosage form, liquid dosage form or as a dissolved solution, the in vivo exposure (or bioavailability) of BH4 is higher when ingested just after meals compared to fasting controls.

The BH4 and the food may be ingested at approximately the same time, or the BH4 may be ingested before or after the food. The period of time between consuming the food and taking BH4, either swallowed or dissolved, may be at least 5 minutes. For example, BH4 may be administered 60 minutes, 30 minutes, 25 minutes, 20 minutes, 15 minutes, 10 minutes, or 5 minutes before or after a meal.

In another embodiment, for some patients, e.g. adults, or some disease states, e.g. cardiovascular diseases or other diseases associated with NOS dysfunction, the methods of the invention involve administering an intact tablet rather than dissolving the tablet in a liquid, in order to improve bioavailability.

In a second aspect, the invention contemplates a method of stabilizing BH4 in a patient's intestinal tract by decreasing intestinal pH, e.g. using proton exchange polymers. Corresponding products comprising BH4 and acidifying excipients, such as proton exchange polymers, are also contemplated.

A third aspect of the invention contemplates a method of increasing gut residence time for BH4, including but not limited to slowing of gut motility using an agent which slows gut motility, such as a fatty acid and/or a glycerol fatty acid ester. Such hydrophobic agents can increase the length of time that BH4 remains in the gut and can increase the amount of BH4 that gets absorbed. The length of time that BH4 remains in the gut, when formulated with such agent(s), can be at least one and a half times, at least two times, at least three times, at least four times, or at least five times longer than a BH4 formulation not having such an agent. Suitable fatty acids include oleic acid, stearic acid, arachidic acid, palmitic acid, archidoic acid, linoleic acid, linolenic acid, erucic acid, myristic acid, lauric acid, myristolic acid, and palmitolic acid. Also contemplated to increase gut residence time for BH4 is inducement of gastric retention using alginic acid, and bioadhesion using polycarbophil. Corresponding products comprising BH4 and agents that slow gut motility are contemplated.

A fourth aspect of the invention contemplates a method of modifying the release of BH4 using a sustained release for-

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mulation such as HPMC, carbomer, etc. Corresponding products that are sustained release formulations are contemplated.

In a fifth aspect, the invention contemplates administering BH4 in sterile liquid or sterile solid dosage form via routes other than oral administration including but not limited to topical, intravenous, subcutaneous, intramuscular, intrathecal, ophthalmic, and inhalational routes of administration. Corresponding compositions and kits suitable for such routes of administration, and methods of making the same, are contemplated. For example, a transdermal or buccal patch for transdermal or buccal administration, respectively, comprising BH4 is contemplated. Sublingual tablets comprising BH4 are also contemplated. Suitable kits are contemplated, including an inhaler device comprising BH4, or a kit comprising BH4 and a dropper or sprayer.

One embodiment includes a liquid formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, including an aqueous solution of BH4 or pharmaceutically acceptable salt thereof, an antioxidant, and a pH buffer.

Another embodiment includes a method of making a liquid formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, including providing an aqueous solution containing BH4 or pharmaceutically acceptable salt thereof, adding an antioxidant and a pH buffer to the solution containing BH4 or pharmaceutically acceptable salt thereof, sparging the aqueous solution containing BH4 or pharmaceutically acceptable salt thereof, before or after addition of antioxidant and pH buffer, with an inert gas or carbon dioxide, and sealing the sparged solution containing BH4 or pharmaceutically acceptable salt thereof, antioxidant, and pH buffer in a container.

In a sixth aspect, the invention contemplates an improved method of measuring BH4 by utilizing tandem mass spectrometry and calculating the amount of reduced biopterin. Such methods can provide detection of BH4 to a sensitivity for BH4 in the range of 5-1000 ng/mL, with an accuracy and precision as exemplified by a coefficient of variation (CV) % below 15% (20% at the lower limit of quantitation, LLOQ). In an exemplary embodiment, a method of measuring BH4 using HPLC (RP) coupled with tandem mass spectrometry (LC/MS/MS) comprises the steps of: (1) subjecting samples of blood, plasma, tissue homogenates, or urine to oxidation; (2) subjecting the oxidized samples to iodometry; (3) passing said oxidized samples through an ion exchange column; (4) measuring total and oxidized biopterin in said samples using HPLC and tandem mass spectrometry; and calculating the amount of reduced biopterin as the difference between said total biopterins less said oxidized form. In one embodiment, samples are treated with acidic oxidation, wherein the method comprises the steps of (1) treating said samples with KCl, HCl or TCA; (2) subjecting said acid-oxidized samples to iodometry; (3) running said oxidized samples through an ion exchange column; (4) measuring total biopterin comprising 6R-BH4, R-q-DHBP (which is immediately reduced in vivo to 6R-BH4 such that the measured reduced biopterin is based mainly upon 6R-BH4), DHBP, and BP in said samples using HPLC and tandem mass spectrometry. In another embodiment, samples are treated by alkaline oxidation, wherein the method comprises: (1) treating said samples with KI, I or NaOH; (2) subjecting said alkaline oxidized samples to acidification with HCl or TCA; (3) subjecting said oxidized samples iodometry; (4) running said samples through an ion exchange column; (5) measuring oxidized biopterin comprising DHBP and BP using HPLC and tandem mass spectrometry; and (6) calculating the amount of reduced biopterin (6R-BH4+R-q-DHBP) as the difference between total biopterins less the oxidized form.

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Another aspect of the invention is a mobile phase solution for reverse-phase HPLC separation of dihydrobiopterin, biopterin, and analogs thereof, including an aqueous solution including methanol, sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol. Similarly contemplated is a method of separating dihydrobiopterin and biopterin, or analogs thereof, from a mixture containing both base and dihydro forms, including performing reverse phase HPLC using a mobile phase comprising an aqueous solution including methanol, sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol, on a mixture containing dihydrobiopterin and biopterin, or an analog of dihydrobiopterin and an analog of biopterin.

Another aspect of the invention is a method of quantitating biopterins in a mixture of biopterin species, including providing a mixture comprising biopterin and at least one of dihydrobiopterin and tetrahydrobiopterin, or analogs of biopterin and at least one of dihydrobiopterin and tetrahydrobiopterin, separating the biopterin species in the mixture by reverse phase HPLC, and in the case of tetrahydrobiopterin and analogs thereof, performing electrochemical detection by oxidizing the tetrahydrobiopterin and analogs thereof present by a first electrode to quinonoid dihydrobiopterin forms, followed by reducing the quinonoid forms back to tetrahydrobiopterin and analogs thereof present at a second electrode, and measuring current generated by the reduction reaction to determine the concentration of species, and/or in the case of dihydrobiopterin, analogs thereof, biopterin, or analogs thereof, measuring such species by fluorescence detection following post-column oxidation of dihydrobiopterin species to biopterin.

For the compositions and methods described herein, preferred components, and compositional ranges thereof, can be selected from the various examples provided herein.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a powder X-ray diffraction pattern characteristic of crystal polymorph form B of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin.

FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

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FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 16 is a flow chart for the measurement of biopterin.

FIG. 17 is a summary of the validation of the biopterin assay.

FIG. 18 is a table showing pharmacokinetic parameters of total biopterins in plasma after a single oral administration of sapropterin (BH4) to rats.

FIG. 19 shows plasma biopterin concentration and reduced-form ratio after single-dose administration of sapropterin (BH4) to rats.

FIG. 20 shows plasma biopterin concentration and reduced-form ratio after a single-dose administration of sapropterin (BH4) in monkeys.

FIG. 21 is a table showing pharmacodynamic parameters of total biopterins in plasma after single-dose administration of sapropterin (BH4) to monkeys.

FIG. 22 shows the schedule of events for the evaluation of safety.

FIG. 23 shows the mean plasma concentrations of BH4 after oral administration of 10 mg/kg of BH4 as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers—linear axes.

FIG. 24 shows the mean plasma concentrations of BH4 after oral administration of 10 mg/kg of BH4 as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers—semi-logarithmic axes.

FIG. 25 shows a table summarizing the pharmacokinetic parameters for BH4 after oral administration of 10 mg/kg of BH4 as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers.

FIG. 26 shows a statistical comparison of pharmacokinetic parameters for BH4 after oral administration of 10 mg/kg of BH4 as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers.

FIG. 27 shows a stability study of BH4 formulated with 5% mannitol in an aqueous solution both before and after two weeks stored at -20° C.

FIG. 28 shows a dissolution profile of a BH4 capsule formulation both before and after storage for 54 days at 40° C.

FIG. 29 shows a dissolution profile of two BH4 formulations—a BH4 bioadhesive tablet and BH4 bioadhesive granules.

FIG. 30 shows a dissolution profile of various sustained release formulations of BH4.



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FIG. 31 shows a dissolution profile of various sustained release formulations of BH4.

FIG. 32 shows a schematic diagram of a floating dosage formulations of BH4.

FIG. 33 shows a dissolution profile of various floating dosage formulations.

FIG. 34 shows a schematic diagram of gas generating dosage forms of BH4.

FIG. 35 shows a pharmacokinetic profile of various BH4 formulations.

FIG. 36 shows a stability study of intravenous BH4 formulations at pH 4 over 35 days.

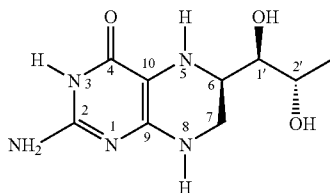
FIG. 37 shows a stability study of various intravenous BH4 formulations over 350 hours.

FIG. 38 shows a stability study of intravenous BH4 formulations at various BH4 concentrations.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides improved methods of orally administering a purified preparation of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin, including a pharmaceutically acceptable salt thereof. The invention is based on the finding that orally administered tetrahydrobiopterin (BH4) has low gastrointestinal absorption, which is a major contributing factor to the low bioavailability of BH4.

The chemical structure of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4) is shown below:



Tetrahydrobiopterin is a water soluble organic compound with low lipid solubility. Based on an in silico experimental analysis using BioLoom software (version 1.5 from Biobyte Corp in Claremont Calif.), the octanol-water partition coefficient of BH4 was determined to be -1.17. Optimal penetration of biological membranes as approximated by the octanol/water partition coefficient occurs at around a log P of 2 or 100- $\times$  fold higher lipid solubility. Although a low ClogP allows this substrate to solubilize readily under physiological conditions, the ability of the substrate to penetrate bilipid layers within biological membranes is restricted, which may limit oral availability.

In vivo studies in rats and monkeys described herein showed that only 8-11% of BH4 is absorbed in the gut with the majority being excreted in the feces when compared to intravenous administration of BH4 at similar doses. Such variability in absorption of BH4 was also shown in a study described herein on the effect of food on the bioavailability of BH4 in healthy humans. Although the administration of BH4 in water and orange juice under fasted conditions resulted in comparable mean plasma concentrations and mean values for Cmax and AUC(0-t), the administration of BH4 concurrent with a high fat, high caloric meal resulted in a significant increase in the mean plasma concentrations and mean values for Cmax and AUC(0-t) when BH4 was administered in water.

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Although there is ample literature describing increased bioavailability in fed conditions, this food effect is typically seen with lipophilic (i.e., lipid soluble) water-insoluble drugs and not usually with high water soluble active substance such as BH4. The usual explanation for increases in bioavailability under fed conditions for lipophilic compounds is that high fat meals help solubilize the drug since "like dissolves like" and this makes it available for absorption. Another possible explanation is that high fat meals stimulate the secretion of bile acids which are natural bio-surfactants that help solubilize and emulsify the fats we eat to aid their digestion. These bile acids are also thought to solubilize water-insoluble compounds thereby making them available for absorption. However, BH4 does not need solubilization to be absorbed since its solubility is greater than 1000 mg/mL and the compound is one of the most soluble drugs known. Therefore the enhancement of its bioavailability by high fat, high-energy meals is not consistent with such known mechanism.

However, administration as a solid or semi-solid dosage form and/or with a high-fat meal may maximize bioavailability by increasing the residence time of BH4 in the acidic milieu of the stomach and upper gastrointestinal tract (GIT) where BH4 is chemically stable. The stability of BH4 decreases with increasing pH and its half-life in pH 6.8 buffer solution, which is roughly the pH of the small intestine, is about 15 minutes. At pH 3.1, which is within realm of the typical pH of the stomach in normal volunteers, the stability of BH4 at a concentration of 1 mg/mL is over 3 hours. The chemical stability of BH4 may further increase when the pH of the stomach drops below pH 3.1. Therefore prolonged stomach residence time provides intact drug to the stomach wall for absorption, whereas rapid emptying into the intestine degrades BH4 and is thus not available to be absorbed.

Thus, to maximize oral bioavailability of BH4 at each administration, BH4 should be taken with food, e.g., a high fat food or a high fat and/or high calorie meal. Alternatively, to maximize consistency of oral bioavailability between administrations, BH4 should be taken on an empty stomach (e.g., 1 hour before or 2 hours after a meal).

As used herein, the term "bioavailability" refers to the fraction of an administered dose of a drug entering systemic circulation. If the drug were administered intravenously, then its bioavailability theoretically would be 100%. However, if the drug were administered via other routes (such as orally), then its bioavailability would be less than 100% as a result of, for example, incomplete absorption in the GI tract, degradation or metabolism prior to absorption, and/or hepatic first pass effect.

The term "high fat meal" refers generally to a meal of at least about 700 kcal and at least about 45% fat (relative percentage of kcal which are fat), or alternatively at least about 900 kcal and at least about 50% fat. The term "high fat food" refers generally to a food comprising at least 20 g of fat, or at least 25, 30, 35, 40, 45, or 50 g of fat, and/or at least about 45% or 50% fat. One FDA Guidance defines a "high-fat meal" as approximately 50% of total caloric content of the meal, whereas a "high-calorie meal" is approximately 800 to 1000 calories. The FDA recommends a high-fat and high-calorie meal as a test meal for food-effect bioavailability and fed bioequivalence studies. This test meal should derive approximately 150, 250, and 500-600 calories from protein, carbohydrate and fat, respectively. An example test meal consists of two eggs fried in butter, two strips of bacon, four ounces of hash brown potatoes and eight ounces of whole milk. Substitution is possible if a similar amount of calories from protein, carbohydrate, and fat has comparable meal volume and viscosity (Guidance for Industry, Food-Effect



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Bioavailability and Fed Bioequivalence Studies, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), December 2002).

In a first aspect, the invention provides methods of orally administering a purified preparation of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4), including a pharmaceutically acceptable salt thereof.

In some embodiments, the methods involve informing the patient that administration of tetrahydrobiopterin with food has an effect on pharmacokinetics. In an exemplary embodiment, the methods comprise the step of informing the patient that absorption of tetrahydrobiopterin is increased when it is ingested with food compared to when ingested without food. In some embodiments, the patient is informed that ingestion shortly following a meal, for example, a high-fat, high-calorie meal, results in an increase in any one, two, three or all of the following parameters: mean plasma concentration, C<sub>max</sub>, AUC, AUC(0-t) and/or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 with a high-fat meal increases C<sub>max</sub> and AUC compared to administration of BH4 without food (in a fasting condition). In some embodiments, the relative increase can be at least 20% or 30% or more.

In alternative embodiments or in addition to the preceding embodiments, the method of administering tetrahydrobiopterin comprises informing the patient that absorption of tetrahydrobiopterin is increased when ingested as an intact tablet compared to when ingested after being dissolved in liquid. In some embodiments, the patient is informed that ingestion of intact tablets results in an increase in any of the following parameters: mean plasma concentration, C<sub>max</sub>, AUC, AUC(0-t) or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 as an intact tablet increases C<sub>max</sub> and AUC compared to administration of BH4 after being dissolved in a liquid. In some embodiments, the relative increase can be at least 20% or more.

Any of the preceding methods may be carried out by providing or administering tetrahydrobiopterin in a container containing printed labeling informing the patient of the change in absorption parameters described above.

Optionally, the methods of the invention also comprise the step of providing to the patient in need thereof a therapeutically effective amount of tetrahydrobiopterin. The therapeutically effective amount will vary depending on the condition to be treated, and can be readily determined by the treating physician based on improvement in desired clinical symptoms.

In one exemplary embodiment, such methods involve administering BH4 in a dissolved form, wherein the formulation is dissolved in a liquid including but not limited to water, orange juice and apple juice. In one embodiment, dissolved BH4 is administered to the patient in a fasting condition, i.e., on an empty stomach. The invention further contemplates that the dissolved BH4, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal. Thus, BH4 may be ingested as a liquid product or pre-dissolved from a solid or semisolid dosage form prior to ingestion. In a further embodiment, BH4 may also be dissolved in the oral cavity from a solid or semisolid dosage form prior to swallowing the dissolved solution.

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These approaches maximize absorption rate and bioavailability by ensuring that BH4 is fully dissolved in solution or biologic fluids before it is delivered to its absorption sites, which are primarily the stomach and the intestine. Dissolution of active pharmaceutical ingredients or drug in solution is a prerequisite to absorption into the systemic (blood and lymphatic) circulation. When solid dosage forms such as tablets and capsules are administered orally, they go through a sequential series of steps such as disintegration into granules, de-aggregation into powders and dissolution prior to absorption into the systemic circulation. These series of steps are bypassed by administering liquid, semisolid and fast dissolving solid dosage forms. Thus the active substance is available earlier for absorption, and because there is no guarantee that a solid dosage form will release all the active substance contained within it before it transits through the absorptive sites, the formulations in which the active substance is present in dissolved form before it reaches the absorptive sites usually exhibits the greater bioavailability.

These dosage forms reduce variability in blood levels because the variability is dosage form disintegration and dissolution in vivo in the human is obviated. The rate of in vivo disintegration and dissolution of a solid dosage form of BH4 targeted for immediate-release in the stomach depends on the human-to-human variability in the pH of the gastric fluid—fed and unfed (fasting)—and the strength of the agitation intensity of the stomach as determined by the strength of gastric motility and gastric emptying rates into the small intestine. Since liquid, semisolid, lozenge/candy and fast dissolving solid dosage forms do not have to be subjected to disintegration and dissolution, their blood levels are less variable than when BH4 is given as immediate release solid dosage forms (tablets and capsules).

In another exemplary embodiment, such methods involve administering BH4 in a solid dosage form including but not limited to tablets, capsules, candies, lozenges, powders, and granules, or semisolid form, including but not limited to oral sprinkle into jelly, that is chewed or swallowed without dissolving in a liquid including but not limited to water, orange juice and apple juice, before swallowing. In one embodiment, swallowed BH4 is administered to the patient in a fasting condition, i.e., on an empty stomach. The invention further contemplates that the BH4 swallowed as a solid or semisolid dosage form, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, 2.5 hours, or three hours after a meal.

In another embodiment, such methods involve administering BH4, whether swallowed as a solid or semisolid dosage form, or dissolved in a liquid, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal. The invention further contemplates that BH4, whether swallowed or dissolved, is administered at a specified time including but not limited to morning, day, night, same time of the day, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal, one or more times a day. In an exemplary embodiment, BH4 is ingested once daily as a solid dosage form just after meals. In a preferred embodiment the solid dosage form is a formulated tablet or capsule. In more exemplary embodiments, BH4 is ingested within approximately 0 to 60 minutes, approximately 0 to 30, or 5 to 20 minutes of eating a meal. Regardless of whether it is ingested as a solid dosage form, liquid dosage form or as a dissolved solution, the in vivo exposure (or

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bioavailability) of BH4 is higher when ingested just after meals compared to fasting controls.

The BH4 and the food may be ingested at approximately the same time, or the BH4 may be ingested before or after the food. The period of time between consuming food, e.g., a high-fat food or a high-fat and/or high-calorie meal and taking BH4 either swallowed or dissolved may be at least 5 minutes. BH4 may be administered 60 minutes, 30 minutes, 25 minutes, 20 minutes, 15 minutes, 10 minutes, or 5 minutes after ingestion of a meal.

In another embodiment, for some patients, e.g. adults, or some disease states, e.g. cardiovascular diseases or other diseases associated with NOS dysfunction, the methods of the invention involve administering an intact tablet rather than dissolving the tablet in a liquid, in order to improve bioavailability.

Administration of BH4 according to the methods of the invention results in mean plasma concentrations and/or rate of gastrointestinal absorption and/or mean values for Cmax and/or AUC(0-t) and/or AUC (inf) that exceeds the values when BH4 is administered under fasted conditions.

Administration of an intact tablet under fasted conditions resulted in an average 20% increase in Cmax and AUC relative to dissolved tablets. Administration of a dissolved tablet in either water or orange juice or an intact tablet after a high fat/high calorie meal resulted in increases in Cmax and AUC that ranged from approximately 30% (intact tablet) to 80% (water). Administration of BH4 as an intact tablet following a high fat and high calorie meal resulted in an approximate 30% increase in the extent of absorption compared to administration without food. Administration of BH4 as an intact tablet resulted in an approximate 20% increase in the extent of absorption compared to administration of dissolved tablets.

"Mean plasma concentration" means the average of readings of concentration in a series of plasma samples.

"Cmax" means the maximum observed plasma concentration.

"AUC" means the area under the plasma concentration-time curve.

"AUC<sub>0-t</sub>" means the area under the plasma concentration-time curve from time 0 to the time of the last measurable concentration.

"AUC<sub>(inf)</sub>" means the calculated area under the plasma concentration-time curve from time 0 to infinity.

The "rate of gastrointestinal absorption" of BH4 is estimated from the area under the plasma total biopterin concentration increase (ΔCp)-time curve (ΔAUC) after the administration of BH4 using the following formula:

$$\text{Absorption rate(\%)} = (\Delta\text{AUC after p.o. dose} / \Delta\text{AUC after i.v. dose}) \times (\text{i.v. dose} / \text{p.o. dose} \times 100)$$

Preferably at least 99.5% pure 6R-BH4 is used. Any salt, including the dihydrochloride salt, and any crystalline form of BH4 may be utilized according to the methods and compositions of the invention. A variety of salts and crystalline forms are described in U.S. Patent Publication No. 2006/0040946, incorporated herein by reference in its entirety, and/or the stable solid formulation described in Int'l Publication No. WO 06/55511, also incorporated herein by reference in its entirety. The various crystalline forms may conveniently be formed into a tablet, powder or other solid for oral administration.

In a second aspect, the invention contemplates a method of stabilizing BH4 by decreasing intestinal pH using proton exchange polymers. BH4 is administered orally daily as a solid or liquid dosage form comprising inactive ingredients that enhance the stability of BH4 beyond the stomach by

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lowering the pH of the intestine and thus preserving BH4 from being oxidized rapidly. Since BH4 is more stable in acidic media than in basic media, acidifying excipients/inactive ingredients are included in solid dosage (tablets, capsules, etc) formulations of BH4 to lower the pH of the intestinal fluids and thereby enhance the chemical stability. The larger area or window of the gastrointestinal tract (GIT) available for absorption optimizes the consistency of absorption by expanding the current limited window of absorption believed to be limited to the stomach and the duodenum to the intestine. Such dosage forms include but are not limited to effervescent tablets, powders and granules (to be resuspended in liquid before administration) and acidifier materials. Unlike small molecule acids, bulky polymeric acids remain in the GIT longer and are not absorbed by the GIT, but donate their protons to the GIT fluids to lower the environmental pH. Examples of excipients/inactive ingredients that comprise the formulation are carboxylic acid small molecules such as maleic, fumaric and citric acids or inorganic small molecules such as phosphoric acid, acetic acid and their salt forms. Other examples are pharmaceutically acceptable acids such as polymeric carboxylic acid classes including polymethacrylic acids, carbomers, polycarbophil, Eudragits, acid forms of crosscarmellose and starch glycolic acid, etc. The formulations also contain additional excipients to enhance stability such as antioxidants (e.g., thiols such as cysteine, N-acetyl cysteine, etc.; ascorbic acid; methionine; etc.) and other excipients known in the trade to enable manufacturability and enhance the quality and performance attributes of the formulation.

A third aspect of the invention contemplates a method of increasing gut residence time for BH4, including but not limited to slowing of gut motility using an agent which is capable of slowing gut motility of BH4, such as a fatty acid and/or a glycerol fatty acid ester. Fatty acids can include oleic acid, stearic acid, arachidic acid, palmitic acid, archidoic acid, linoleic acid, linolenic acid, erucic acid, myristic acid, lauric acid, myristolic acid, and palmitolic acid. Also contemplated for increasing gut residence time of BH4 are the inducement of gastric retention using alginic acid and bioadhesion using polycarbophil. In one embodiment, dosage forms of BH4 are administered as oral buoyant formulations that float and release BH4 in a defined fashion in the gastric fluid and are retained longer in the stomach because they are more resistant to gastric emptying from the stomach than formulations that are non-buoyant or dissolve rapidly in the stomach. This design approach is based on gastro-retention of the dosage form via the use of a gas-generating excipient within the dosage form, low-density excipients that render the dosage form buoyant in GIT fluids or a combination of a gas and low-density materials in a dosage form to enable the floating of the dosage form in the fluid contents of the GIT. Prolonged retention and release of the dosage form in the stomach milieu wherein BH4 is more stable in its acidic fluids will enhance both residence time of the dosage form in the stomach and the stability of BH4 and thus make BH4 available for a longer period absorption in the stomach and duodenum than standard tablet and capsule dosage forms. Formulations of BH4 will comprise of one or more antioxidants, excipients known in the field to enable manufacturing and disintegration/dissolution of the solid dosage form and additional excipients that generate a gas or mixture of gases (e.g., carbon dioxide) upon contact of the formulation with aqueous media and or GIT fluids. Water-soluble antioxidants are preferred, for example, ascorbic acid, methionine, and thiols (cysteine, N-acetyl cysteine and glutathione) or anti-oxidants that are converted to a soluble antioxidant in the GIT, e.g.,

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ascorbyl palmitate which is converted to ascorbic acid in the GIT. Excipients added to the formulation include carbonates and bicarbonates that react directly with BH4 to form carbon dioxide and small and polymeric acids described previously to react with the carbonates and bicarbonates to produce additional carbon dioxide as needed.

In another embodiment, dosage forms of BH4 are administered that adhere for a prolonged time to the mucous surfaces of the GIT (i.e., bioadhesive formulation), preferably in, but by no means limited to the stomach where due to the acidity of gastric fluids, BH4 is more stable than in the intestine. BH4 is released in a controlled manner from the bioadhesive dosage form. The solid dosage form is designed to contain BH4, one or more antioxidants, excipients known in the field to enable the manufacturing of quality dosage forms and control the disintegration/dissolution of the dosage form and a bioadhesive additive such as polycarbophil in its free acid form or as a salt form. Other polymeric acids such as polymethacrylic acids, carbomers and cellulose derivatives, e.g., HPMC, HPC, etc. may be combined with or substituted for polycarbophil. The antioxidants are preferably soluble, for example, ascorbic acid, methionine, cysteine, N-acetyl cysteine and glutathione or can be converted to a soluble antioxidant such as ascorbic acid in the GIT, e.g., ascorbyl palmitate. In one embodiment, the components of the formulation are blended together and manufactured as a solid dosage form, e.g., tablets or capsules. The solid dosage form may be enteric coated to deliver BH4 past the stomach into the intestine or not enteric coated designed to release BH4 in the stomach. In another embodiment, the components of the solid dosage form may be subdivided into different portions and the various portions are blended separately before they are processed to form multilayered dosage forms. The multilayered dosage form may contain the bioadhesive and a few excipients in the outermost layer of a tablet, wrapped around other layers that contain BH4 (i.e., active region inside a bioadhesive envelope) or as a wrap-around cylindrical plug filled into a capsule wherein one or more other layers are assembled beneath or within the bioadhesive envelope. Alternatively, the bioadhesive and other layers in the tablet or capsule plugs may be layered in a parallel bi- or multilayer configuration. These designs allow the bioadhesive to interact with the GI membrane or GI membrane mucus to anchor the dosage form to the membrane slowing down its transit through the GI tract and thus increasing residence time. Such dosage forms may also be enteric coated. Yet another embodiment of the method used with BH4 is to employ polymeric inactive ingredients (excipients) with functional groups that bind to GIT mucus to delay the transit of the dosage form through the GIT. Dosage forms of BH4 are formulated with thiolated polymer excipients (polymer-SH) such as polycarbophil-cysteine, polypolymethacrylic acid-cysteine, carboxymethyl cellulose-cysteine, chitosan derivatives-cysteine, etc. These thiolated polymers confer both bioadhesive and anti-oxidant properties on BH4 considerably enhancing absorption. Other excipients included in these formulations are antioxidants and performance and manufacture-aiding excipients.

In yet another embodiment, oral dosage forms containing inactive excipients or active ingredients are used to slow gastric motility. Slowing down the transit of BH4 dosage form through the GIT tract will increase the residence time of the molecule and thus enable a larger fraction of the administered dose to be absorbed. Generally regarded as safe (GRAS) excipients employed in oral formulations to delay gastric emptying and/or delay intestinal motility preferably comprise dietary fats such as fatty acids, glycerides of fatty

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acids, and derivatives of fatty acids and glycerides such as Cremophor™ (polyoxyl castor oil derivatives), etc. Active excipients include agents that slow gut motility such as general or selective ( $M_3$ ) antimuscarinic or anticholinergic agents.

A fourth aspect of the invention contemplates a method of modifying the release of BH4 using a sustained release formulation such as HPMC, carbomer, etc. This concept comprises delivering BH4 dosage forms to the GI tract by modifying or altering the release of BH4 from immediate-release to slow, prolonged, controlled and or timed release. Slow, prolonged and controlled release is achieved using excipients known in the art and BH4 is protected within the delivery system from chemical degradation by the presence of stability enhancers such as anti-oxidants. Such methods can maximize bioavailability since BH4 is stabilized within the formulation and in the environment surrounding the formulation to enable the active molecule to be absorbed intact into the systemic circulation as the formulation transits the entire length of the GIT. This approach provides a larger window of the GIT for absorption and does so by preventing the degradation of BH4 in the higher pH milieu so that BH4 is available to be absorbed. Antioxidants will be included in the formulation to protect the drug from degrading in intestinal fluids due to near neutral pH of the intestinal fluids. Slow, prolonged and controlled delivery will also deliver BH4 to low oxygen tension regions of the GIT. Timed release is achieved using excipients known in the art such as pH sensitive polymers that dissolve only when the pH reaches a value wherein the polymer is soluble.

In another embodiment, the invention contemplates enteric coating of the BH4 dosage form to ascertain whether including acidic excipients in a formulation of BH4 does indeed increase absorption of BH4 by lowering the pH of the intestine and thus stabilizing BH4 in the intestine to be available for absorption. Thus, enteric coating will be used to keep the excipients and drug together at the site where the excipient is expected to protect BH4. If the BH4 dosage form were allowed to disintegrate in the stomach, the acidic excipients may not empty together into the stomach and may not provide protection.

Enteric coating protects compounds susceptible to acid-catalyzed degradation in the stomach from getting degraded by the acid in the stomach. Enteric coating materials prevent the tablet or capsule from releasing the active compound in the stomach because the enteric coating materials are insoluble in acid. Once the enteric-coated dosage form reaches the intestine where the pH value varies from pH 5-8, the materials become soluble and release the active substance in the intestine. In contrast, sustained release formulations are designed to release medicaments over as long a length/area of the GIT as possible. Coating a sustained release formulation to release just past the stomach may be necessary only if the medicaments contained in it are acid-labile.

In a fifth aspect, the invention contemplates administering BH4 in sterile liquid or sterile solid dosage form via routes other than oral administration including but not limited to topical, intravenous, subcutaneous, intramuscular, intrathecal, ophthalmic, and inhalational routes of administration. BH4 is formulated as a sterile liquid or solid dosage form at the appropriate concentration desired.

The advantages of a sterile liquid dosage form of BH4 for intravenous administration may include: (1) more predictable kinetics, with the potential for higher serum levels; (2) no requirement of a functional gastrointestinal tract; (3) no requirement for patient participation; and (4) absence of a noncompliance concern. Intravenous formulations of BH4



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may be particularly beneficial in managing conditions requiring expedited delivery of fluids and medications throughout the body or to body compartments normally difficult to access via oral or other forms of administration, including but not limited to rabies, meningitis, organ transplantation/preservation, sub-arachnoidal hemorrhages, brain trauma, stroke, coronary artery bypass surgery, cerebrovascular vasospasm, blood transfusion/preservation, pulmonary hypertension, sickle cell disease, pre-eclampsia, and post-chemotherapy vascular disease.

BH4 is highly susceptible to oxidation in aqueous solution and in physiologic aqueous pH solutions (Davis, et al., Eur. J. Biochem. 173, 345-351 (1988); Kirsch, et al., J. Biol. Chem. 278, 24481-24490 (2003)). Most determinations of BH4 stability have been carried out in neutral to mildly alkaline pH 7.4 solutions to mimic the likely stability behavior of BH4 under physiologic plasma pH condition. Although European Patent Application No. 1 757 293 A discloses liquid or syrup formulations, such formulations consist of solid state powder blends or granulations that require reconstitution with water prior to oral ingestion. The present aspect of invention contemplates liquid formulations not limited to powders or granulations for constitution. The invention also contemplates compounded liquid formulations able to remain stable at ambient temperature for a sufficient period of time to allow processing in sterile product fill/finish facilities to be filled into ampoules, bottles or vials as a liquid product or filled into vials to be freeze-dried into lyophilized products.

The liquid and lyophilized formulations for reconstitution can also be delivered via the nasal, ophthalmic and ear canal for therapeutic effects. The formulation of a lyophilized product requires prior dissolution of BH4 in a liquid, preferably aqueous, and the processing of the liquid product in a sterile facility (i.e. compounding, sterile filtration and filling of the sterile-filtered liquid into vials prior to the loading of the filled vials into a lyophilizer for lyophilization). Maintaining the stability of solubilized BH4 during sterile processing and preventing its degradation are key prerequisites to manufacturing lyophilized product that satisfies impurities specification for the fill-finished product. Therefore the composition of the lyophilized product contains appropriate stabilizers that minimize or obviate BH4 degradation during the fill finish process. The formulations described herein would stabilize BH4 solutions during sterile fill/finish manufacturing, a process that takes a minimum of six hours, and also provide commercially stable product.

The formulations include BH4, preferably in concentration in a range of 0.1 mg/mL to 10 mg/mL. Due to the high solubility of BH4, formulations with concentrations up to about 100 mg/mL, for example, can also be prepared. The general relative compositional makeup and methods described herein are applicable for making highly concentrated solutions.

Liquid formulations of BH4 preferably are formulated in pH 1 to 8 buffer solutions, preferably in pH 2 to 7 buffer solutions. The pH buffers chosen are buffer compounds capable of providing substantial buffering capacity at a particular pH desired, as judged by how close the buffer ionization constant or constants are to the desired pH of the liquid formulation. Thus any buffer compounds may be employed as long as one or more of the compound's ionization constants are close to the desired pH of the formulation. Examples of buffers that may be employed in the pH 1-8 range comprise various acids/bases and their respective conjugate acids/bases or salt forms, including but not limited to: hydrochloric acid (pH 1-2), maleic acid (pH 1-3), phosphoric acid (pH 1-3),

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citric acid (pH 3-6), acetic acid (pH 4.7±1.0), sodium phosphate dibasic (pH 6-8), tromethamine (TRIS, pH 8.3±1.0), and the like.

#### Intravenous Formulations

Intravenous formulations are stabilized using an anti-oxidant or a combination of 2 or more antioxidants. Combinations of anti-oxidants can be synergistic in obviating instability of the formulation. Sparging with inert gases and/or carbon dioxide to remove dissolved oxygen from solution is optional, but is preferred when low concentrations of antioxidants are used, and further preferably when both low concentrations of BH4 and antioxidants are used. Stabilization of BH4 in aqueous solution is influenced by the interactions of the concentration of BH4 with the antioxidant and pH. Thus, for example, high concentrations of BH4 require less antioxidant concentrations than low concentrations of BH4. Furthermore, BH4 is more stable at low pH than at high pH. Therefore desired high pH formulations preferably have higher antioxidant concentrations, more preferably a combination of 2, 3, or more antioxidants, and still further preferably sparging with non-oxidizing gas (e.g., inert gas or carbon dioxide) followed by hermetically or near-hermetically sealing the primary container in an atmosphere of a non-oxidizing gas (e.g., inert gas or carbon dioxide) to further enhance the stability of the drug product.

Example ranges for BH4 liquid formulations are given in Tables 1 and 2. Formulated or compounded solutions are optionally sparged with an inert gas (e.g., argon or nitrogen) or carbon dioxide in the compounding tank and primary containers preferably are sealed in a blanket of inert gas or carbon dioxide to remove oxygen from the container headspace. The formulation can be scaled up to any volume by multiplying the component amounts by an appropriate scale up factor.

TABLE 1

General examples of composition ranges in a low pH (e.g., pH 4.0) formulation

Components	Amount (mg)	% Weight/Volume	Function
BH4	0.10-100	0.01-10.00	Active substance
L-Cysteine	0.00-50.00	0.00-5.00	Antioxidant
Ascorbic Acid	0.00-500.00	0.00-50.00	Antioxidant
Sodium Metabisulfite	0.00-300.00	0.00-30.00	Antioxidant
Citric Acid	0.26-19.87	0.03-1.99	Buffering agent
Sodium Citrate, Dihydrate	2.57-192.75	0.26-19.27	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 2

General examples of composition ranges of a neutral pH (e.g., pH 7.0) formulation

Components	Amount (mg)	% Weight/Volume	Function
BH4	0.10-100	0.01-10.00	Active substance
L-Cysteine	0.00-50.00	0.00-5.00	Antioxidant
Ascorbic Acid	0.00-500.00	0.00-50.00	Antioxidant
Sodium Metabisulfite	0.00-300.00	0.00-30.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	0.50-11.02	0.05-1.02	Buffering agent
Sodium Dibasic Phosphate	0.44-17.80	0.04-1.78	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

The antioxidants employed for liquid formulations preferably are selected from one or more of thiol-based (e.g., L-cysteine), ascorbic acid and sulfite-based (e.g. sodium met-

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abisulfite) compounds. Solutions preferably are sparged with inert gases or carbon dioxide to expel oxygen from the BH4 solutions and then hermetically sealed in ampoules or hermetically capped vials and bottles using metallic beverage beer-type caps in a blanket of inert gases (e.g., argon, nitrogen) or non-inert gas such as carbon dioxide to keep the sparged gases in the container head spaces from escaping. Oral liquid formulations preferably additionally contain sweeteners and flavorants improve the palatability of the formulations.

In one embodiment, as a liquid dosage form, BH4 is stabilized by anti-oxidants and/or by sparging with non-oxidizing, preferably sterilized, gases, such as inert gasses (e.g., nitrogen, argon, helium, etc.) and/or a non-inert gas such as carbon dioxide to remove molecular oxygen from the formulation. The product is preferably filled under a blanket of inert gasses to minimize or prevent molecular oxygen from redissolving in the formulation. The liquid is filled into a container (e.g., vials, ampoules, etc.) and hermetically sealed to prevent oxygen from entering the container. In another embodiment, as a sterile solid dosage form for parenteral administration, a solution of BH4 is lyophilized and reconstituted in the clinic prior to administration. In yet another embodiment, sterile powder drug substance of BH4 is directly packaged into sterile containers (e.g., vials, bags, bottles or ampoules) in a sterile dry powder fill facility. Thus, another aspect of the invention is a dry powder formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof for constitution into an aqueous solution, including a dry powder mixture of BH4 or pharmaceutically acceptable salt thereof, an antioxidant, and a pH buffer.

#### Oral Liquid Formulation Compositions

Oral liquid formulations comprise in addition to the components employed in the general liquid and intravenous formulations, sweeteners and flavoring agents. Sweeteners and flavors are added in quantities sufficient to yield acceptable sweetness and flavor. Oral liquid formulations contain one or more stabilizers. Optionally, they contain antimicrobial preservatives. They are preferentially buffered at low pH e.g., pH 1-4 and the buffering agents are selected to match the flavoring agent thus enhancing the organoleptic properties of the oral liquid formulation. Examples of preferred buffers (acid and conjugate bases) are: citric acid, tartaric acid, malic acid in combination with their conjugate bases or salt forms.

Examples of sweeteners include sugars (e.g., sucrose, glucose, sorbitol, mannitol, fructose, etc.), intense non-sugar sweeteners (e.g., aspartame, acesulfame K, cyclamate, saccharin, sucralose, glycyrrhizin, alitame, neotame, neohesperidine DC, thaumatin, monellin, and the like).

In a further embodiment, for nasal, ophthalmic and otic administrations, BH4 is formulated as discussed for parenteral dosage forms and is optionally a sterile product. These dosage forms can be provided in a kit package presentation with several days of supplies. Each unit within the kit can be comprised of one vial or ampoule and one sprayer (for nasal dosage form) or one dropper (in the case of ophthalmic and otic dosage forms). Once the vial or ampoule is opened, the sprayer or dropper is screwed onto the vial or ampoule and the previous cap is discarded. The dosage form product is used within a prescribed expiration period and discarded and a new vial or ampoule is opened for use. Another embodiment is to fill the solutions in hermetic plastic single-use disposable sterile containers produced by a form-fill-and-seal manufacturing process. These packages are opened and the solutions delivered using the desired route of administration by squeezing out the liquid contained within them. These dosage forms are administered once daily and are given via the nostrils

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(nasal product), or via the eyes (ophthalmic) or droplets are instilled into the auditory canal (otic product). With respect to medication packaged in form, a fill and seal package, the medication is squeezed out onto the route of administration.

In a further embodiment, BH4 is administered via buccal and transdermal routes using formulated strips, patches or films or as topical products that placed on the site of delivery. Sublingual tablets are placed beneath the tongue. These dosage forms are administered once daily and are either attached to the delivery site membrane (buccal and transdermal route) or placed as a solid or semi-dosage form in the sublingual site. To prevent irritation of the delivery site, a basic compound such as sodium carbonate or bicarbonate is coated and mixed with BH4 to prevent interaction with BH4 that would render it unstable. Alternatively the basic compound is added just before use to raise the pH of BH4, which is quite low. Adding the basic excipient at the time of manufacturing without coating the alkaline particles to prevent interaction with BH4, will lead to instability of BH4. Another embodiment is to coat a core sublingual tablet of BH4 with a coating solution containing a basic or alkaline substance. In the sublingual compartment, the basic compound dissolves first, and interacts with BH4 to raise the pH of the medium.

#### Primary Container Packaging for BH4 Liquid Formulations

The primary packaging containers for BH4 liquid formulations are preferably impermeable to oxygen, carbon dioxide, nitrogen and inert gases. Following filling of sparged liquid formulations of BH4 into the primary container, preferably under a blanket of nitrogen, the containers are preferably hermetically sealed to keep the sparging gas in the liquid and container headspace and prevent the loss of the sparging gas and ingress of oxygen into the container.

The preferred primary containers are hermetically sealed ampoules as well as bottles and vials sealed hermetically with metallic cap such as those employed in sealing soda and beer beverage bottles. During use, the ampoules are cut opened and used within a few hours, e.g., about 12 hours. Ampoules can be used for intravenous and sterile products for injections. Sterile injectable liquids and lyophilized products can also be packaged in rubber closure-sealed vials which are secured with crimped aluminum cap. The antioxidants in the formulations protect the liquid and lyophilized products from the imperceptibly slow loss of sparged gas or oxygen ingress into the vial for the shelf life of the product.

BH4 liquid formulations filled into bottles or vials for oral, ophthalmic or otic use preferably are hermetically secured with a beverage metallic cap or a rubber stopper secured with crimped aluminum seal. The flutes of the bottles or vials can be grooved to accept a screw cap. When the hermetic seal is removed, it is replaced with a screw cap with or without a dropper. The presence of antioxidants in the formulation can enable the screw-capped formulation to be stable for use for at least two weeks, for example, after the hermetic seal is broken.

#### I. SYNTHESIS OF TETRAHYDROBIOPTERIN

A variety of methods are known in the art for synthesis of tetrahydrobiopterins, precursors, derivatives and analogs. U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408, Canadian application CA 2420374, European application nos. EP 079 574, EP 191 335 and Suntory Japanese patent publications JP 4-082888, JP 59-021685 and JP 9-157270, as well as Sugimoto and Mats-

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uura, *Bull. Chem. Soc. Japan*, 48(12):3767-3768 (1975), Sugimoto and Matsuura, *Bull. Chem. Soc. Japan*, 52(1):181-183 (1979), Matsuura et al., *Chem. Lett. (Japan)*, 735-738 (1984), Matsuura et al., *Heterocycles*, Vol. 23, No. 12, 3115-3120, 1985 and Whiteley et al., *Anal Biochem.* 137(2):394-6 (1984) (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivatives thereof that may be used as compositions for the present invention.

Int'l Publication No. WO2005049614, U.S. Pat. No. 4,540, 783, Japanese Patent No. 59-021685, Schircks et al., *Helv. Chim. Acta*, 60: 211 (1977), Sugimoto et al., *Bull. Chem. Soc. Jp.* 52(1):181 (1979), Sugimoto et al., *Bull. Chem. Soc. Jp.* 48(12):3767 (1975), Visontini et al., *Helv. Chim. Acta*, 52:1225 (1969), and Matsuura et al., *Chem. Lett.*, p 735 (1984), incorporated herein by reference in their entireties, describe methods of synthesizing BH4.

## II. CRYSTALLINE FORMS OF 6R-TETRAHYDROBIOPTERIN HYDROCHLORIDE SALT

(6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists in different crystalline forms, including polymorphic forms and solvates, some of which are more stable than others.

Crystal Polymorph Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

### Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B." Results obtained during investigation and development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride development revealed that there are several known crystalline solids have been prepared, but none have recognized the polymorphism and its effect on the stability of the BH4 crystals.

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties render polymorph form B especially feasible for pharmaceutical application, which are prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

As used herein, the following the abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity. A characteristic X-ray powder diffraction pattern is exhibited in FIG. 1.

It has been found that other polymorphs of BH4 have a satisfactory chemical and physical stability for a safe handling during manufacture and formulation as well as providing a high storage stability in its pure form or in formulations. In addition, it has been found that form B, and other polymor-

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phs of BH4 can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive of crystal form B, can be used for the preparation of the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms than polymorph form B, such as polymorph A, in suitable polar and non aqueous solvents. Thus, the pharmaceutical preparations described herein refer to a preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Other forms of BH4 can be converted for form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methyl-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallization from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may then be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water:acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallization from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallization and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter



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isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties render polymorph form B especially feasible for pharmaceutical application.

#### Polymorph Form A

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form A," or "polymorph A." Polymorph A is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph A is a hygroscopic anhydrate, which is a meta-stable form with respect to form B; however, it is stable over several months at ambient conditions if kept in a tightly sealed container. Form A is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form A can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Polymorph A which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) of: 15.5 (vs.), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m), 3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w). FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph A exhibits a characteristic Raman spectra bands, expressed in wave numbers (cm<sup>-1</sup>) at: 2934 (w), 2880 (w), 1692 (s), 1683 (m), 1577 (w), 1462 (m), 1360 (w), 1237 (w), 1108 (w), 1005 (vw), 881 (vw), 813 (vw), 717 (m), 687 (m), 673 (m), 659 (m), 550 (w), 530 (w), 492 (m), 371 (m), 258 (w), 207 (w), 101 (s), 87 (s) cm<sup>-1</sup>.

Polymorph form A may be obtained by freeze-drying or water removal of solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water. Polymorph form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at ambient temperatures in water, (1) cooling the solution to low temperatures for solidifying the solution, and removing water under reduced pressure, or (2) removing water from said aqueous solution.

The crystalline form A can be isolated by filtration and then dried to evaporate absorbed water from the product. Drying conditions and methods are known and drying of the isolated product or water removal pursuant to variant (2) described herein may be carried out in applying elevated temperatures,

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for example up to 80° C., preferably in the range from 30° C. to 80° C., under vacuum or elevated temperatures and vacuum. Prior to isolation of a precipitate obtained in variant (2), the suspension may be stirred for a certain time for phase equilibration. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 5 to 40 percent by weight, by reference to the solution.

A fast cooling is preferred to obtain solid solutions as starting material. A reduced pressure is applied until the solvent is completely removed. Freeze drying is a technology well known in the art. The time to complete solvent removal is dependent on the applied vacuum, which may be from 0.01 to 1 mbar, the solvent used and the freezing temperature.

Polymorph form A is stable at room temperature or below room temperature under substantially water free conditions, which is demonstrated with phase equilibration tests of suspensions in tetrahydrofuran or tertiary-butyl methyl ether stirred for five days and 18 hours respectively under nitrogen at room temperature. Filtration and air-drying at room temperature yields unchanged polymorph form A.

#### Polymorph Form F

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form F," or "polymorph F." Polymorph F is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph F is a meta-stable form and a hygroscopic anhydrate, which is more stable than form A at ambient lower temperatures and less stable than form B at higher temperatures and form F is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form F can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Polymorph F exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 17.1 (vs.), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w), 3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 2.96 (m). FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph F may be obtained by phase equilibration of suspensions of polymorph form A in suitable polar and non-aqueous solvents, which scarcely dissolve said lower energy forms, especially alcohols such as methanol, ethanol, propanol and isopropanol. Polymorph form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can also be prepared by dispersing particles of solid form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-aqueous solvent that scarcely dissolves said (6R)-L-erythro-tetrahydrobiopterin dihydrochloride below room temperature, stirring the suspension at said temperatures for a time sufficient to produce polymorph form F, thereafter isolating crystalline form F and removing the solvent from the isolated form F. Removing of solvent and drying may be carried out under air, dry air or a dry protection gas such as nitrogen or noble gases and at or below room temperature, for example down to 0° C. The temperature during phase equilibration is preferably from 5 to 15° C. and most preferably about 10° C.



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## Polymorph Form J

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form J,” or “polymorph J.” The polymorph J is slightly hygroscopic and adsorbs water when handled at air humidity. The polymorph J is a meta-stable form and a hygroscopic anhydrate, and it can be transformed back into form E described below, from which it is obtained upon exposure to high relative humidity conditions such as above 75% relative humidity. Form J is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form J can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form J exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs). FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph J may be obtained by dehydration of form E at moderate temperatures under vacuum. In particular, polymorph form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by taking form E and removing the water from form E by treating form E in a vacuum drier to obtain form J at moderate temperatures, which may mean a temperature in the range of 25 to 70° C., and most preferably 30 to 50° C.

## Polymorph Form K

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form K,” or “polymorph K.” Polymorph K is slightly hygroscopic and adsorbs water to a content of about 2.0 percent by weight, which is continuously released between 50° C. and 100° C., when heated at a rate of 10° C./minute. The polymorph K is a meta-stable form and a hygroscopic anhydrate, which is less stable than form B at higher temperatures and form K is especially suitable as intermediate and starting material to produce stable polymorph forms, in particular form B. Polymorph form K can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form K exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m). FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph K may be obtained by crystallization from mixtures of polar solvents containing small amounts of water and in the presence of small amounts of ascorbic acid. Solvents for the solvent mixture may be selected from acetic acid and an alcohol such as methanol, ethanol, n- or isopropanol. In particular, polymorph form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and an alcohol or tetrahydrofuran containing small amounts of water and a small amount of ascorbic acid at elevated temperatures, lowering temperature

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below room temperature to crystallize said dihydrochloride, isolating the precipitate and drying the isolated precipitate at elevated temperature optionally under vacuum. Suitable alcohols are for example methanol, ethanol, propanol and isopropanol, whereby ethanol is preferred. The ratio of acetic acid to alcohol or tetrahydrofuran may be from 2:1 to 1:2 and preferably about 1:1. Dissolution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be carried out in presence of a higher water content and more of the anti-solvent mixture can be added to obtain complete precipitation. The amount of water in the final composition may be from 0.5 to 5 percent by weight and the amount of ascorbic acid may be from 0.01 to 0.5 percent by weight, both by reference to the solvent mixture. The temperature for dissolution may be in the range from 30 to 100 and preferably 35 to 70° C. and the drying temperature may be in the range from 30 to 50° C. The precipitate may be washed with an alcohol such as ethanol after isolation, e.g., filtration. The polymorph K can easily be converted in the most stable form B by phase equilibration in e.g., isopropanol and optionally seeding with form B crystals at above room temperature such as temperatures from 30 to 40° C.

## Hydrate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline hydrate, which shall be described and defined herein as forms C, D, E, H, and O. These hydrate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

## Hydrate Form C

It has been found that a hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form C,” or “hydrate C.” The hydrate form C is slightly hygroscopic and has a water content of approximately 5.5 percent by weight, which indicates that form C is a monohydrate. The hydrate C has a melting point near 94° C. ( $\Delta H_f$  is about 31 J/g) and hydrate form C is especially suitable as intermediate and starting material to produce stable polymorphic forms. Polymorph form C can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form C exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m). FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form C may be obtained by phase equilibration at ambient temperatures of a polymorph form such as polymorph B suspension in a non-solvent, which contains water in an amount of preferably about 5 percent by weight, by reference to the solvent. Hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by suspending (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-solvent such as, heptane, C1-C4-alcohols such as methanol, ethanol, 1- or 2-propanol, acetates, such as ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or binary or ter-

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nary mixtures of such non-solvents, to which sufficient water is added to form a monohydrate, and stirring the suspension at or below ambient temperatures (e.g., 0 to 30° C.) for a time sufficient to form a monohydrate. Sufficient water may mean from 1 to 10 and preferably from 3 to 8 percent by weight of water, by reference to the amount of solvent. The solids may be filtered off and dried in air at about room temperature. The solid can absorb some water and therefore possess a higher water content than the theoretical value of 5.5 percent by weight. Hydrate form C is unstable with respect to forms D and B, and easily converted to polymorph form B at temperatures of about 40° C. in air and lower relative humidity. Form C can be transformed into the more stable hydrate D by suspension equilibration at room temperature.

## Hydrate Form D

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form D," or "hydrate D." The hydrate form D is slightly hygroscopic and may have a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form D is a monohydrate. The hydrate D has a melting point near 153° C. ( $\Delta H_f$  is about 111 J/g) and is of much higher stability than form C and is even stable when exposed to air humidity at ambient temperature. Hydrate form D can therefore either be used to prepare formulations or as intermediate and starting material to produce stable polymorph forms. Polymorph form D can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form D exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w). FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form D may be obtained by adding at about room temperature concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents, and stirring the suspension at ambient temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding at about room temperature a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non-solvent from 1:10 to 1:1000. Form D contains a small excess of water, related to the monohydrate, and it is believed that it is absorbed water due to the slightly hygroscopic nature of this crystalline hydrate. Hydrate form D is deemed to be the most stable one under the known hydrates at ambient temperatures and a relative humidity of less than 70%. Hydrate form D may be used for formulations prepared under conditions, where this hydrate is stable. Ambient temperature may mean 20 to 30° C.

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## Hydrate Form E

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form E," or "hydrate E." The hydrate form E has a water content of approximately 10 to 14 percent by weight, which suggests that form E is a dihydrate. The hydrate E is formed at temperatures below room temperature. Hydrate form E is especially suitable as intermediate and starting material to produce stable polymorph forms. It is especially suitable to produce the water-free form J upon drying under nitrogen or optionally under vacuum. Form E is non-hygroscopic and stable under rather high relative humidities, i.e., at relative humidities above about 60% and up to about 85%. Polymorph form E can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form E exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w). FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form E may be obtained by adding concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent cooled to temperatures from about 10 to -10° C. and preferably between 0 to 10° C. and stirring the suspension at said temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent, which is cooled to temperatures from about 10 to -10° C., and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non-solvent from 1:10 to 1:1000. A preferred non-solvent is tetrahydrofuran. Another preparation process comprises exposing polymorph form B to an air atmosphere with a relative humidity of 70 to 90%, preferably about 80%. Hydrate form E is deemed to be a dihydrate, whereby some additional water may be absorbed. Polymorph form E can be transformed into polymorph J upon drying under vacuum at moderate temperatures, which may mean between 20° C. and 50° C. at pressures between 0 and 100 mbar. Form E is especially suitable for formulations in semi solid forms because of its stability at high relative humidities.

## Hydrate Form H

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form H," or "hydrate H." The hydrate form H has a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form H is a hygroscopic monohydrate.

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The hydrate form H is formed at temperatures below room temperature. Hydrate form H is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form H can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form H exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 8.6 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w), 3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m). FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form H may be obtained by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water, adding then a non-solvent to precipitate a crystalline solid, cooling the obtained suspension and stirring the cooled suspension for a certain time. The crystalline solid is filtered off and then dried under vacuum at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is tetrahydrofuran. Hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and a less amount than that of acetic acid of water, adding a non-solvent and cooling the obtained suspension to temperatures in the range of  $-10$  to  $10^\circ\text{C}$ ., and preferably  $-5$  to  $5^\circ\text{C}$ ., and stirring the suspension at said temperature for a certain time. Certain time may mean 1 to 20 hours. The weight ratio of acetic acid to water may be from 2:1 to 25:1 and preferably 5:1 to 15:1. The weight ratio of acetic acid/water to the non-solvent may be from 1:2 to 1:5. Hydrate form H seems to be a monohydrate with a slight excess of water absorbed due to the hygroscopic nature.

#### Hydrate Form O

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form O," or "hydrate O." The hydrate form O is formed at temperatures near room temperature. Hydrate form O is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form O can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form O exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m). FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form O can be prepared by exposure of polymorphic form F to a nitrogen atmosphere containing water vapor with a resulting relative humidity of about 52% for about 24 hours. The fact that form F, which is a slightly hygroscopic

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anhydrate, can be used to prepare form O under 52% relative humidity suggests that form O is a hydrate, which is more stable than form F under ambient temperature and humidity conditions.

#### Solvate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline solvate forms, which shall be described and defined herein as forms G, I, L, M, and N. These solvate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

#### Solvate Form G

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form G," or "hydrate G." The ethanol solvate form G has a ethanol content of approximately 8.0 to 12.5 percent by weight, which suggests that form G is a hygroscopic mono ethanol solvate. The solvate form G is formed at temperatures below room temperature. Form G is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form G can be prepared as a solid powder with a desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form G exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w). FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form G may be obtained by crystallization of L-erythro-tetrahydrobiopterin dihydrochloride dissolved in water and adding a large excess of ethanol, stirring the obtained suspension at or below ambient temperatures and drying the isolated solid under air or nitrogen at about room temperature. Here, a large excess of ethanol means a resulting mixture of ethanol and water with less than 10% water, preferably about 3 to 6%. Ethanol form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at about room temperature to temperatures of  $75^\circ\text{C}$ . (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water or in a mixture of water and ethanol, cooling a heated solution to room temperature and down to 5 to  $10^\circ\text{C}$ ., adding optionally ethanol to complete precipitation, stirring the obtained suspension at temperatures of 20 to  $5^\circ\text{C}$ ., filtering off the white, crystalline solid and drying the solid under air or a protection gas such as nitrogen at temperatures about room temperature. The process may be carried out in a first variant in dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at about room temperature in a lower amount of water and then adding an excess of ethanol and then stirring the obtained suspension for a time sufficient for phase equilibration. In a second variant, (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be suspended in ethanol, optionally adding a lower amount of water, and heating the suspension and dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, cooling down the solution to temperatures of about 5 to  $15^\circ\text{C}$ ., adding additional ethanol to the suspension and then stirring the obtained suspension for a time sufficient for phase equilibration.



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## Solvate Form I

It has been found that an acetic acid solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form I," or "hydrate I." The acetic acid solvate form I has an acetic acid content of approximately 12.7 percent by weight, which suggests that form I is a hygroscopic acetic acid mono solvate. The solvate form I is formed at temperatures below room temperature. Acetic acid solvate form I is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form I can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form I exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m), 3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w). FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Acetic acid solvate form I may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water at elevated temperature, adding further acetic acid to the solution, cooling down to a temperature of about 10° C., then warming up the formed suspension to about 15° C., and then stirring the obtained suspension for a time sufficient for phase equilibration, which may last up to 3 days. The crystalline solid is then filtered off and dried under air or a protection gas such as nitrogen at temperatures about room temperature.

## Solvate Form L

It has been found that a mixed ethanol solvate/hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form L," or "hydrate L." Form L may contain 4% but up to 13% ethanol and 0% to about 6% of water. Form L may be transformed into form G when treated in ethanol at temperatures from about 0° C. to 20° C. In addition form L may be transformed into form B when treated in an organic solvent at ambient temperatures (10° C. to 60° C.). Polymorph form L can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form L exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w). FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Form L may be obtained by suspending hydrate form E at room temperature in ethanol and stirring the suspension at temperatures from 0 to 10° C., preferably about 5° C., for a time sufficient for phase equilibration, which may be 10 to 20 hours. The crystalline solid is then filtered off and dried preferably under reduced pressure at 30° C. or under nitrogen. Analysis by TG-FTIR suggests that form L may contain variable amounts of ethanol and water, i.e., it can exist as an polymorph (anhydrate), as a mixed ethanol solvate/hydrate, or even as a hydrate.

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## Solvate Form M

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form M," or "hydrate M." Form M may contain 4% but up to 13% ethanol and 0% to about 6% of water, which suggests that form M is a slightly hygroscopic ethanol solvate. The solvate form M is formed at room temperature. Form M is especially suitable as intermediate and starting material to produce stable polymorph forms, since form M can be transformed into form G when treated in ethanol at temperatures between about -10° to 15° C., and into form B when treated in organic solvents such as ethanol, C3 and C4 alcohols, or cyclic ethers such as THF and dioxane. Polymorph form M can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form M exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs). FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in ethanol and evaporation of the solution under nitrogen at ambient temperature, i.e., between 10° C. and 40° C. Form M may also be obtained by drying of form G under a slight flow of dry nitrogen at a rate of about 20 to 100 ml/min. Depending on the extent of drying under nitrogen, the remaining amount of ethanol may be variable, i.e., from about 3% to 13%.

## Solvate Form N

It has been found that another solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form N," or "hydrate N." Form N may contain in total up to 10% of isopropanol and water, which suggests that form N is a slightly hygroscopic isopropanol solvate. Form N may be obtained through washing of form D with isopropanol and subsequent drying in vacuum at about 30° C. Form N is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form N can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form N exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83 (w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w). FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

The isopropanol form N may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in 4.0 ml of a mixture of isopropanol and water (mixing volume ratio for example 4:1). To this solution is slowly added isopropanol (IPA, for example about 4.0 ml) and the resulting suspension is cooled to 0° C. and stirred for several hours (e.g., about 10 to 18 hours) at this temperature. The suspension is filtered and the solid residue washed with isopropanol at room temperature. The obtained crystalline material is then dried at ambient temperature (e.g., about 20 to 30° C.) and reduced pressure (about 2 to 10 mbar) for several hours (e.g., about 5 to 20 hours). TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water.

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This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as an non-solvated form containing a small amount of water.

For the preparation of the polymorph forms, there may be used crystallization techniques well known in the art, such as stirring of a suspension (phase equilibration in), precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 100° C. may be applied to form solutions. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Meta-stable polymorphs or pseudo-polymorphic forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

It was surprisingly found that hydrate form D is the most stable form under the hydrates and forms B and D are especially suitable to be used in pharmaceutical formulations. Forms B and D presents some advantages like an aimed manufacture, good handling due to convenient crystal size and morphology, very good stability under production conditions of various types of formulation, storage stability, higher solubility, and high bioavailability. Accordingly, one embodiment of the compositions and methods disclosed herein is pharmaceutical composition including polymorph form B and/or hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

### III. PHARMACEUTICAL FORMULATIONS

The formulations described herein are preferably administered as oral formulations. Oral formulations are preferably solid formulations such as capsules, tablets, pills and troches, or liquid formulations such as aqueous suspensions, elixirs and syrups. The various form of BH4 described herein can be directly used as powder (micronized particles), granules, suspensions or solutions, or it may be combined together with other pharmaceutically acceptable ingredients in admixing the components and optionally finely divide them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches, or suspend or dissolve them in carriers for suspensions, elixirs and syrups. Coatings may be applied after compression to form pills.

Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be, for example, binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that are approved by the U.S. Food and Drug Administration or a corresponding foreign regulatory agency for administration to humans. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

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The initial amount of (6R)-L-erythro-tetrahydrobiopterin used to prepare the formulation may be, for example, in the range of about 30 wt % to about 40 wt % of the formulation, or in the range of about 32 wt % to about 35 wt %, or at about 33 wt %. Specific amounts of BH4 in a formulation contemplated herein include 80 mg, 100 mg, 200 mg, 300 mg, 400 mg, and 500 mg.

Binders assist in maintaining a solid formulation. In some cases, anhydrous binders are used to preserve the anhydrous state of polymorph forms. In some cases, the binder may act as a drying agent. Exemplary binders include anhydrous dibasic calcium phosphate and its monohydrate. Other nonlimiting examples of binders useful in a composition described herein include gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (poly-oxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Disintegration agents assist in rapid disintegration of solid formulations by absorbing water and expanding. Exemplary disintegration agents include polyvinylpyrrolidone (PVP, e.g. sold under the name POVIDONE), a cross-linked form of povidone (CPVP, e.g. sold under the name CROSPOVIDONE), a cross-linked form of sodium carboxymethylcellulose (NaCMC, e.g. sold under the name AC-DI-SOL), other modified celluloses, and modified starch. Tablets formulated with CPVP exhibited much more rapid disintegration than tablets formulated with PVP.

Antioxidants may be included and help stabilize the tetrahydrobiopterin product, especially after dissolution. Low pH aqueous solutions of API are more stable than are solutions at neutral or high pH. Antioxidants are included in a formulation described herein to prevent deterioration from oxidation. Antioxidants can generally be classified into 3 groups.

The first group is known as true antioxidants, and inhibit oxidation by reacting with free radicals blocking the chain reaction. Examples include phenolic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl-hydroquinone (TBHQ), 4-hydroxymethyl-2,6-di-tert-butylphenol (HMBP), and 2,4,5-trihydroxybutyrophenone (THBP); alkylgallates, including propyl gallate; gallic acid; nordihydroguaiaretic acid; and tocopherols, including alpha-tocopherol.

The second group, consisting of reducing agents, have lower redox potentials than the drug which they are intended to protect, and are therefore more readily oxidized. Reducing agents may act also by reacting with free radicals. Examples include ascorbic acid, thioglycolic acid (TGA), ascorbyl palmitate, sulfites, including potassium and sodium salts of sulphurous acid (e.g., potassium sulfite, sodium sulfite, sodium metabisulphite, and sodium bisulfite), and thioglycerol.

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The third group consists of antioxidant synergists which usually have a modest antioxidant effect themselves but probably enhance the action of antioxidants in the first or second group by reacting with heavy metal ions which catalyze oxidation. Examples of such antioxidant synergists and chelating agents include citric acid, malic acid, editic acid and its salts, lecithin, and tartaric acid.

Exemplary acidic antioxidants include ascorbic acid, fatty acid esters of ascorbic acid such as ascorbyl palmitate and ascorbyl stearate, and salts of ascorbic acid such as sodium, calcium, or potassium ascorbate. Non-acidic antioxidants may also be used in the stable tablet formulations. Nonlimiting examples of non-acidic antioxidants include beta-carotene, alpha-tocopherol. Acidic additives may be added to enhance stability of the tablet formulation, including citric acid or malic acid. Small molecule anti-oxidants include but are not limited to thiols, e.g., cysteine, N-acetyl cysteine, glutathione, etc., or thiolated polymers (polymer-SH), e.g., polycarbophil-cysteine, polymethacrylic-SH, carboxy methylcellulose-cysteine, etc. or small molecule anti-oxidants such as ascorbic acid, methionine, ascorbyl palmitate, etc. These anti-oxidants confer stability on the dosage form during transit through the GIT, particularly as the pH of the GIT increases with distance from the stomach.

In one embodiment, a combination of at least two reducing agent antioxidants is preferred. In another embodiment, a combination of at least two reducing agent antioxidants together with an acid antioxidant synergist and/or chelating agent is preferred.

Lubricants improve stability, hardness and uniformity of solid formulations. Exemplary lubricants include stearyl fumarate and magnesium stearate. Other nonlimiting examples of lubricants include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Optionally the stable formulations of the invention can also comprise other excipients such as mannitol, hydroxyl propyl cellulose, microcrystalline cellulose, or other non-reducing sugars such as sucrose, trehalose, melezitose, planteose, and raffinose. Reducing sugars may react with BH4. Other non-limiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate.

Surfactants for use in a composition described herein can be anionic, anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange

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flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The BH4 form may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. Slow release formulations may also be prepared in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used. Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N' dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4 aminosalicic acid, 2 phenoxybenzoic acid, 2 acetoxymethoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzenesulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2



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sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

Exemplary stable oral formulations contain one or more of the following additional ingredients that improve the stability or other characteristics of the formulation: binder, disintegration agent, acidic antioxidant, or lubricant or combinations thereof. Exemplary stable tablet formulations include a binder and disintegration agent, optionally with an acidic antioxidant, and optionally further including a lubricant. Exemplary concentrations of binder are between about 1 wt % to about 5 wt %, or between about 1.5 and 3 wt %; an exemplary weight ratio of binder to BH4 is in the range of about 1:10 to about 1:20. Exemplary concentrations of disintegration agent are between about 1 wt % to about 20 wt %; an exemplary weight ratio of disintegration agent to BH4 is in the range of about 1:5 to about 1:10. Exemplary concentrations of antioxidant are between about 1 wt % and about 3 wt %; an exemplary weight ratio of antioxidant to BH4 is in the range of about 1:5 to 1:30. In one example, ascorbic acid is the antioxidant and is used at a ratio to BH4 of less than 1:1, e.g. 1:2 or less, or 1:10 or less. Exemplary concentrations of lubricant in a stable tablet formulation of the present invention are between about 0.1 wt % and about 2 wt %; an exemplary weight ratio of lubricant to BH4 is in the range of about 1:25 to 1:65.

The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or arginine; and/or vitamins, such as vitamin C and/or vitamin B2 (riboflavin) and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan.

Exemplary folates, including folate precursors, folic acids, or folate derivatives, are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and pharmaceutically acceptable salts thereof. Exemplary salts include sodium, potassium, calcium or ammonium salts.

Exemplary relative weight ratios of BH4 to folates to arginine may be from about 1:10:10 to about 10:1:1.

The stable formulations of the invention may be provided, e.g. as tablets or pills or capsules in HDPE bottles provided with a desiccant capsule or pouch; or in foil-on-foil blister

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packaging, or in blister packaging comprising see-through polymer film, if commercially desirable.

#### IV. TREATMENT OF BH4-RESPONSIVE DISEASES

##### Hyperphenylalaninemia, Neuropsychological or Neuropsychiatric Disorders

The methods of the invention may be used for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine or tryptophan levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal.

Conditions associated with decreased tyrosine or tryptophan levels include neurotransmitter deficiency, neurological and psychiatric disorders such as Parkinson's, dystonia, spinocerebellar degeneration, pain, fatigue, depression, other affective disorders and schizophrenia. NO overproduction by nNOS has been implicated in strokes, migraine headaches, Alzheimer's disease, and with tolerance to and dependence on morphine. BH4 may be administered for any of these conditions. Other exemplary neuropsychiatric disorders for which BH4 may be administered include Parkinson's disease, Alzheimer's disease, schizophrenia, schizophreniform disorder, schizoaffective disorder, brief psychotic disorder, delusional disorder, shared psychotic disorder, psychotic disorder due to a general medical condition, substance-induced psychotic disorder, other psychotic disorders, tardive dyskinesia, Machado-Joseph disease, spinocerebellar degeneration, cerebellar ataxia, dystonia, chronic fatigue syndrome, acute or chronic depression, chronic stress syndrome, fibromyalgia, migraine, attention deficit hyperactivity disorder, bipolar disease, and autism.

The stable formulations may also be used for treating patients suffering from BH4 deficiency, e.g., due to a defect in the pathway for its synthesis, including but not limited to dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, or dihydropteridine reductase (DHPR) deficiency.

Suitable subjects for treatment with the stable formulations of the invention include subjects with an elevated plasma Phe concentration in the absence of the therapeutic, e.g. greater than 1800  $\mu\text{M/L}$ , or greater than 1600  $\mu\text{M}$ , greater than 1400  $\mu\text{M}$ , greater than 1200  $\mu\text{M}$ , greater than 1000  $\mu\text{M}$ , greater than 800  $\mu\text{M}$ , or greater than 600  $\mu\text{M}$ , greater than 420  $\mu\text{M}$ , greater than 300  $\mu\text{M}$ , greater than 200  $\mu\text{M}$ , or greater than 180  $\mu\text{M}$ . Mild PKU is generally classified as plasma Phe concentrations of up to 600  $\mu\text{M/L}$ , moderate PKU as plasma Phe concentrations of between 600  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$  and classic or severe PKU as plasma Phe concentrations that are greater than 1200  $\mu\text{M/L}$ . Preferably treatment with the stable formulations alone or with protein-restricted diet decreases the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ , or less than 500  $\mu\text{M}$ , or 360  $\mu\text{M} \pm 15 \mu\text{M}$  or less, or less than 200  $\mu\text{M}$ , or less than 100  $\mu\text{M}$ . Other suitable subjects include subjects diagnosed as having a reduced phenylalanine hydroxylase (PAH) activity, atypical or malignant phenylketonuria associated with BH4 deficiency, hyperphenylalaninemia associated with liver disorder, and hyperphenylalaninemia associated with malaria. Reduced PAH activity may result from a mutation in the PAH enzyme, for



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example, a mutation in the catalytic domain of PAH or one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C; or subjects that are pregnant females, females of child-bearing age that are contemplating pregnancy, or infants between 0 and 3 years of age, or 0-2, 0-1.5 or 0-1; or subjects diagnosed as unresponsive within 24 hours to a single-dose BH4 loading test or a multiple dose loading test, such as a 4-dose or 7-day loading test. Exemplary patient populations and exemplary BH4 loading tests are described in Int'l. Publication No. WO 2005/049000, incorporated herein by reference in its entirety.

U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment as described herein.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg×5 kg body weight) to 3000 mg/day (30 mg/kg×100 kg body weight). While continuous, daily administration is contemplated, for HPA it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again. Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data.

In exemplary embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

#### Low Dose Regimens

In a low dose therapeutic method of the invention, low doses, e.g., doses of 0.1 to 5 mg/kg per day are contemplated, including doses of 0.1 to 2 mg/kg, or 0.1 to 3 mg/kg, or 1 mg/kg to 5 mg/kg. Doses of less than 5 mg/kg per day are preferred. According to the invention, such doses are expected to provide improvements with relevant study endpoints, and BH4 derivatives are expected to have improved biological properties relative to natural BH4 at such doses. In particular, the invention contemplates that any of the 1',2'-diacyl-(6R,S)-5,6,7,8-tetrahydro-L-biopterins or lipoidal tetrahydrobiopterins described herein exhibit improved biological properties at low doses.

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The invention also specifically contemplates the use of BH4, or a precursor or derivative thereof, for treating BH4-responsive diseases at a dose in the range of 0.1 to 5 mg/kg body weight/day, via any route of administration including but not limited to oral administration, in a once daily dose or multiple (e.g. 2, 3 or 4) divided doses per day, for a duration of at least 1, 2, 3, or 4 weeks or longer, or 1, 2, 3, 4, 5, 6 months or longer. Exemplary doses include less than 5 mg/kg/day, 4.5 mg/kg/day or less, 4 mg/kg/day or less, 3.5 mg/kg/day or less, 3 mg/kg/day or less, 2.5 mg/kg/day or less, 2 mg/kg/day or less, 1.5 mg/kg/day or less, 1 mg/kg/day or less, or 0.5 mg/kg/day or less. Equivalent doses per body surface area are also contemplated.

For the person of average weight/body surface area (e.g. 70 kg), the invention also contemplates a total daily dose of less than 400 mg. Exemplary such total daily doses include 360 mg/day, 350 mg/day, 300 mg/day, 280 mg/day, 210 mg/day, 180 mg/day, 175 mg/day, 150 mg/day, or 140 mg/day. For example, 350 mg/day or 175 mg/day is easily administrable with an oral dosage formulation of 175 mg, once or twice a day. Other exemplary total daily doses include 320 mg/day or less, 160 mg/day or less, or 80 mg/day or less. Such doses are easily administrable with an oral dosage formulation of 80 or 160 mg. Other exemplary total daily doses include 45, 90, 135, 180, 225, 270, 315 or 360 mg/day or less, easily administrable with an oral dosage formulation of 45 or 90 mg. Yet other exemplary total daily doses include 60, 120, 180, 240, 300, or 360 mg/day, easily administrable with an oral dosage formulation of 60 or 120 mg. Other exemplary total daily doses include 70, 140, 210, 280, or 350 mg/day, easily administrable with an oral dosage formulation of 70 or 140 mg. Exemplary total daily doses also include 55, 110, 165, 220, 275 or 330 mg/day, easily administrable with an oral dosage formulation of 55 mg. Other exemplary total daily doses include 65, 130, 195, 260, or 325 mg/day, or 75, 150, 225, 300 or 375 mg/day, e.g. in dosage formulations of 65 mg or 75 mg.

#### Diseases Associated with Nitric Oxide Synthase Dysfunction

The invention further contemplates that stable formulations of the invention may be used for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from co-treatment with folates, including folate precursors, folic acids, or folate derivatives.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (nar-

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rowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases or disorders having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, include but are not limited to abnormal vascular compliance, endothelial dysfunction and hypertension, disorders of insulin sensitivity and glucose control, abnormal peripheral perfusion such as intermittent claudication, reduced peripheral perfusion, decreased skin blood flow, defective wound healing and peripheral circulation disorder, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, coronary arteriostenosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arteriosclerotic heart diseases, congestive heart failure, pulmonary hypertension with or without congestive heart failure, exercise-associated angina, coronary artery disease and related atherosclerosis; ophthalmic disease such as optic atrophy and diabetic retinal disease; and renal disease such as microalbuminuria in diabetic renal disease, renal failure and decreased glomerular filtration rate.

It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

The invention provides a method for treating a subject diagnosed as having vascular disease unrelated to diabetes selected from the group consisting of pulmonary vascular disease, hemolytic anemias, stroke and related ischemic vascular disease (such as stroke, cardiac or coronary disease, arteriosclerosis, or peripheral vascular disease), thrombosis, transplant-related endothelial dysfunction, and cardiac or coronary disease. In one embodiment, pulmonary vascular disease includes but is not limited to pulmonary tension in sickle cell anemia and other hemoglobinopathies, idiopathic pulmonary hypertension, persistent pulmonary hypertension of the newborn (PPHN). In a further embodiment, hemolytic anemias include hereditary hemolytic anemias and acquired hemolytic anemia. Hereditary hemolytic anemias include but are not limited to sickle-cell anemia, thalassemia, hemolytic anemia due to G6PD deficiency, pyruvate kinase deficiency, hereditary elliptocytosis, hereditary spherocytosis, hereditary stomatocytosis, hereditary ovalocytosis, paroxysmal nocturnal hemoglobinuria, and hemoglobin SC disease. Acquired hemolytic anemias include but are not limited to microangiopathic hemolytic anemia, idiopathic autoimmune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents or devices (left ventricular assist devices), mechanical heart valves and bypass devices), and secondary immune hemolytic anemia.

In another embodiment, stroke and related ischemic vascular disease includes but is not limited to vasospasm, such as post-stroke cerebrovascular spasm. Thrombosis includes but

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is not limited to thrombogenesis, thrombosis, clotting, and coagulation. In a further embodiment, transplant-related endothelial dysfunction includes but is not limited to vascular dysfunction after solid organ transplantation and cyclosporine A induced endothelial dysfunction. In yet another embodiment, cardiac or coronary disease includes but is not limited to congestive heart failure, vascular dysfunction and angina associated with hypercholesterolemia, and vascular dysfunction and angina associated with tobacco smoking.

BH4 can also prevent or treat other disorders associated with the overproduction of or damage related to reactive oxygen species, including but not limited to sepsis.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

## V. COMBINATION THERAPY

Certain methods of the invention involve the combined use of the stable formulations of the invention and one or more other therapeutic agents.

In such combination therapy, administration of the stable formulations of the invention may be concurrent with or may precede or follow the administration of the second therapeutic agent, e.g. by intervals ranging from minutes to hours, so long as both agents are able to exert their therapeutic effect at overlapping time periods. Thus, the invention contemplates the stable formulations of the invention for use with a second therapeutic agent. The invention also contemplates use of a second therapeutic agent in preparation of a medicament for

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administration with the stable tetrahydrobiopterin, precursor, derivative or analog formulations of the invention.

Tetrahydrobiopterin therapy may be combined with dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. For example, one could administer to the subject the BH4 composition and a low-phenylalanine medical protein composition in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4.

In some embodiments, the protein-restricted diet is one which is supplemented with amino acids, such as tyrosine, valine, isoleucine and leucine. The patient may be co-administered a low-Phe protein supplement, which may include L-tyrosine, L-glutamine, L-carnitine at a concentration of 20 mg/100 g supplement, L-taurine at a concentration of 40 mg/100 g supplement and selenium. It may further comprise the recommended daily doses of minerals, e.g., calcium, phosphorus and magnesium. The supplement further may comprise the recommended daily dose of one or more amino acids selected from the group consisting of L-leucine, L-proline, L-lysine acetate, L-valine, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-tryptophan, L-serine, L-threonine, L-histidine, L-methionine, L-glutamic acid, and L-aspartic acid. In addition, the supplement may be fortified with the recommended daily dosage of vitamins A, D and E. Optionally, the supplement comprises a fat content that provides at least 40% of the energy of the supplement. Such supplements may be provided in the form of a powder supplement or in the form of a protein bar. In certain embodiments, protein-restricted diet comprises a protein supplement and the BH4 is provided in the same composition as the protein supplement.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, for example, with a delay time of only about 1 hour or less. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the

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present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Gent. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4)243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

BH4 may be co-administered for neuropsychological or neuropsychiatric disorders according to the method of the invention with one or more other neuropsychiatric active agents, including antidepressants, neurotransmitter precursors such as tryptophan, tyrosine, serotonin, agents which activate noradrenergic systems, such as lofepramine, desipramine, reboxetine, tyrosine, agents which act preferentially on serotonin, combined inhibitors of both noradrenaline and serotonin uptake, such as venlafaxine, duloxetine or milnacipran, or drugs which are combined inhibitors of both dopamine and noradrenaline reuptake such as bupropion.

In a related embodiment, BH4 is administered with other therapeutic agents commonly used to treat diabetes, vascular disease, hyperlipidemia. Agents used to treat diabetes, include but not limited to agents that improve insulin sensitivity such as PPAR gamma ligands (thiazolidinediones, glitazones, troglitazones, rosiglitazone (Avandia), pioglitazone), stimulators of insulin secretion such as sulphonylureas (gliquidone, tolbutamide, glimepiride, chlorpropamide, glipizide, glyburide, acetohexamide) and meglitinides (meglitinide, repaglinide, nateglinide) and agents that reduce liver production of glucose such as metformin. Agent used to treat vascular disease, include but not limited to endothelin receptor antagonists commonly used for the treatment of hypertension and other endothelial dysfunction-related disorders, such as bosentan, darusentan, enrasentan, tezosentan, atrasentan, ambrisentan sitaxsentan; smooth muscle relaxants such as PDE5 inhibitors (indirect-acting) and minoxidil (direct-acting); angiotensin converting enzyme (ACE) inhibitors such as captopril, enalapril, lisinopril, fosinopril, perindopril, quinapril, trandolapril, benazepril, ramipril; angiotensin II receptor blockers such as irbesartan, losartan, valsartan, eprosartan, olmesartan, candesartan, telmisartan; beta blockers such as atenolol, metoprolol, nadolol, bisoprolol, pindolol, acebutolol, betaxolol, propranolol; diuretics such as hydrochlorothiazide, furosemide, torsemide, metolazone; calcium channel blockers such as amlodipine, felodipine, nisoldipine, nifedipine, verapamil, diltiazem; alpha receptor blockers doxazosin, terazosin, alfuzosin, tamsulosin; and central alpha agonists such as clonidine. Agents used to treat hyperlipidemia, include but not limited to agents that lower LDL such as statins (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin calcium, simvastatin) and nicotinic acid, cholesteryl ester transfer protein inhibitors (such as torcetrapib), agents that stimulate PPAR alpha such as fibrates, gemfibrozil, fenofibrate, bezafibrate, ciprofibrate,



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agents that bind and prevent reabsorption of bile acids and reduce cholesterol levels such as bile acid sequestrants, cholestyramine and colestipol, and cholesterol absorption inhibitors.

BH4 may also be administered with a factor or combination of factors that enhances or normalizes the production of the vasodilator nitric oxide (NO) alone or in combination with a therapeutic agent. In one embodiment, such factor(s) enhances the activity or expression the de novo biosynthesis of BH4 and is selected from the group consisting of guanosine triphosphate cyclohydrolase I (GTPCH1), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase. In a preferred embodiment of the invention, BH4 synthesis is increased by increasing the expression of GTPCH1 expression by the use of any one or more cyclic adenosine monophosphate (cAMP) analogs or agonists including forskolin, 8-bromo cAMP or other agents that function to increase cAMP mediated cell signaling, for example, cytokines and growth factors including interleukin-1, interferon-gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), c-reactive protein, HMG-CoA-reductases (statins like atorvastatin) nerve growth factor (NGF), epidermal growth factor (EGF), hormones including adrenomedullin and estradiol benzoate, and other compounds such as NADPH and NADPH analogs, caffeine, cyclosporine A methyl-xanthines including 3-isobutyl-1-methyl xanthine, theophylline, reserpine, hydrogen peroxide.

One embodiment of invention therefore relates to increasing GTPCH1 levels by inhibiting the degradation of 3'5'-cyclic nucleotides using inhibitors of the eleven phosphodiesterases families (PDE1-11) including PDE1, PDE3, PDE5. The PDE inhibitors of the present invention include Viagra/sildenafil, cialis/tadalafil, vardenafil/levitra, udenafil, 8-Methoxymethyl-IBMX, UK-90234, dexamethasone, hesperetin, hesperidins, Irsogladine, vinpocetine, cilostamide, rolipram, ethyl beta-carboline-3-carboxylate (beta-CCE), tetrahydro-beta-carboline derivatives, 3-O-methylquercetin and the like.

Another embodiment of the invention relates to increasing the levels of BH4 by increasing the levels of BH4-synthesizing enzymes by gene therapy or endothelium-targeted delivery of polynucleotides of the synthetic machinery of BH4. Yet another embodiment of the invention relates to increasing the levels of BH4 by supplementation with BH4-synthesizing enzymes GTPCH1, PTPS, SR, PCD, DHPR and DHFR. It is contemplated that BH4-synthesizing enzymes encompasses all natural and unnatural forms of the enzymes including mutants of the proteins.

Another embodiment of the invention relates to increasing BH4 levels by diverting the substrate 7,8-dihydroneopterin triphosphate towards BH4 synthesizing enzyme PTPS instead of alkaline phosphatase (AP) by inhibiting AP activity. The agents or compounds that inhibit the activity of AP include phosphate analogs, levamisole, and L-Phe. Another embodiment of the invention relates to agents or compounds that inhibit alkaline phosphatase includes the small inhibitory RNA (siRNA), antisense RNA, dsDNA, small molecules, neutralizing antibodies, single chain, chimeric, humanized and antibody fragments to inhibit the synthesis of alkaline phosphatase.

Another embodiment of the invention includes agents or compounds that enhance the activity of catalysts or cofactors needed for the synthesis of enzymes of the de novo synthesis pathway of BH4 synthesis.

Another embodiment of the invention includes agents or compounds that prevent the degradation of the enzymes needed for the synthesis of BH4. Yet another embodiment of

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the invention includes agents or compounds that prevent the degradation of the catalysts needed for the synthesis of BH4 and its synthetic enzymes including GTPCH1, PTPS and SR.

Another embodiment of the invention relates to increasing the levels of BH4 by increasing the reduction of BH2 via the salvage pathway. In vivo, BH4 becomes oxidized to BH2. BH2 which exist as the quinoid form (qBH2) and as the 7,8-dihydropterin which is reduced to BH4 by DHPR and DHFR respectively. One embodiment of the invention relates to increasing the regeneration or salvage of BH4 from BH2 by modulating the activity and synthesis of the enzymes PCD, DHPR and DHFR using agents or compounds that pathway NADPH, thiols, perchloromercuribenzoate, hydrogen peroxide and the like.

Another embodiment of the invention relates to agents that stabilize BH4 by decreasing the oxidation of BH4 using agents or compounds such as antioxidants including ascorbic acid (vitamin C), alpha tocopherol (vitamin E), tocopherols (e.g vitamin A), selenium, beta-carotenes, carotenoids, flavones, flavonoids, folates, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones.

In a further embodiment, such factor(s) may increase the activity or expression of nitric oxide synthase and thereby enhance the generation of NO.

In yet another embodiment, the invention contemplates factors that inhibit the GTPCH feedback regulatory protein, GFRP. An embodiment of the invention relates to agents or compounds that inhibit the binding of BH4 to the GTPCH1/GFRP complex, thereby preventing the feedback inhibition by BH4. Agents or compounds of this invention include competitive inhibitors such as alternate forms of BH4 with altered affinities for the complex, structural analogs etc. Still another embodiment of the invention includes agents or compounds that enhance the binding of L-phenylalanine to GTPCH1/GFRP inducing the synthesis of BH4. Another embodiment of the invention includes agents or compounds that increase the levels of L-Phe such as precursors of L-Phenylalanine, which serves to inhibit the feedback inhibition of GTPCH1 by GFRP and BH4.

Yet another embodiment of the invention relates to agents or compounds that modulate the activity or the synthesis of GFRP. An embodiment of the invention includes agents or compounds that inhibit the activity of GFRP. Another embodiment of the invention includes the use of siRNA, small molecules, antibodies, antibody fragments and the like to inhibit the synthesis of GFRP.

## VI. BIOPTERIN ASSAYS

The concentration of total biopterin and oxidized biopterin in plasma, blood and other tissues are determined based on the method of Fukushima et al (Anal. Biochem. 102:176 (1980)). Biopterin has four different forms including two forms of reduced biopterin, R-tetrahydrobiopterin (BH4) and quinonoid R-dihydrobiopterin (q-BH2) and two forms of oxidized biopterin, dihydrobiopterin (BH2) and biopterin (B). Of these four forms, only the reduced forms of biopterin have coenzymatic activity. Reduced biopterin is converted to B by iodination under acidic conditions, whereas under alkaline conditions, it is converted to pterin. Oxidized biopterin is converted to B by iodination under acidic and alkaline conditions. By taking advantage of this property, the amount of total biopterin is determined upon iodination under acidic conditions and that of oxidized biopterin is determined upon iodination under alkaline conditions, so that the amount of reduced biopterin is calculated from the difference in quantity thereof. When used as a coenzyme, BH4 is converted to

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q-BH2. The q-BH2 is immediately converted to BH4 by dihydropterine reductase or if not reduced, it is oxidized to BH2 or DHPT. Because it is difficult for biopterin to exist in the form of q-BH2 in vivo, the reduced biopterin may well be displaced as BH4.

Plasma and whole blood samples collected are immediately subjected to oxidation with acidic oxidizing solution (0.6N HCl solution in water containing 0.6% potassium iodide (KI), 0.3% iodine (I2) and 0.6N trichloroacetic acid (TCA)) and alkaline oxidizing solution (0.7N sodium hydroxide (NaOH)). Determination of B is performed by HPLC and radioactivity is measured using a liquid scintillation counter.

Measurement of BH4 using Reverse Phase HPLC (RP) Coupled with Tandem Mass Spectrometry (LC/MS/MS): The combined use of reverse phase high performance liquid chromatography (RP) and tandem mass spectrometry (LC/MS/MS) was shown to be selective for BH4 in human plasma, sensitive for BH4 in the range of 5-1000 ng/mL. The method is associated with about 50% conversion of BH4 due to oxidation during collection and storage. Samples are stable for greater than 3 months in dipotassium salt of ethylenediaminetetraacetic acid (K<sub>2</sub>EDTA) plasma. Recovery from the pre-treatment steps is about 75%. The accuracy and precision of the method was determined to have coefficient of variation (CV) % below 15% (20% at the lower limit of quantitation, LLOQ).

The combined use of HPLC and tandem mass spectrometry was shown to be an improvement over HPLC alone in determining the BH4 test article because of: (1) its increased selectivity for drug-BH4 (whereas HPLC measures total biopterin), (2) broader qualitative range, (3) established conversion ration, (4) extensive characterization and proven utility in human subjects, and (5) novel and useful measurement in different species and matrices.

The improved method comprises the following steps. Samples of blood, plasma, tissue homogenates, or urine are subjected to acidic or alkaline oxidation. With acidic oxidation, (1) the samples are treated with potassium chloride (KCl), hydrochloric acid (HCl) or TCA for an hour; (2) the acid oxidized samples are then subjected to iodometry; (3) the samples are run through an ion exchange column; (4) total biopterin comprising BH4, q-BH2 (which is immediately reduced in vivo to BH4 such that the measured reduced biopterin is based mainly upon BH4), BH2, and B are measured using HPLC and tandem mass spectrometry. With alkaline oxidation, (1) the samples are treated with KI, I2 or NaOH for an hour; (2) the alkaline oxidized samples are then subjected to acidification with HCl or TCA; (3) subjected to iodometry; (4) the samples are run through an ion exchange column; (5) oxidized biopterin comprising BH2 and B are measured; (6) different species are measured using HPLC and tandem mass spectrometry; and (7) the amount of reduced biopterin (BH4+q-BH2) is calculated as the difference between total biopterins less the oxidized form.

Flow charts for biopterin measurement and assay validation summary are provided in FIGS. 16 and 17.

#### Optimized Assay

An HPLC method using Electrochemical Detection (ECD) and Fluorescence (FL) detection is advantageous as it allows for the measurement of each of the discrete biopterin compounds (BH4, BH2 and B) as well as analogs.

BH4 is a cofactor for the enzyme system nitric oxide synthase (NOS), which produces nitric oxide (NO). The production of NO is important for maintaining vascular homeostasis. When intracellular levels of BH4 are limited, NO production is diminished (due to decreased NOS activity) and leads to the

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generation of the damaging free radical superoxide (O<sub>2</sub><sup>-</sup>). Excess O<sub>2</sub><sup>-</sup> can lead to endothelial dysfunction and may contribute to the oxidation of BH4 to BH2. A low ratio of BH4 to BH2 may promote endothelial injury, whereas a high BH4 to BH2 ratio may promote endothelial health. Therefore, characterizing the BH4 to BH2 ratio may serve as a predictor of endothelial health.

The concentrations of different biopterins (BH4, BH2 and B) or analogs are determined by initially using reverse phase HPLC for separation, followed by ECD and FL detection.

BH4, which is a redox-sensitive, non-fluorescent molecule, is measured using ECD. BH4 (and analogs thereof) are measured using ECD in which BH4 (or analog) is oxidized by electrode 1 to a quinonoid dihydrobiopterin form (e.g., qBH2), a short-lived dihydrobiopterin intermediate, which is then reduced back to BH4 (or analog) at electrode 2. The detector then uses the current generated by this reduction reaction to determine the concentration of BH4 or analog thereof (endogenous qBH2 is negligible).

BH2, B, and analogs thereof can be measured in the same injection by fluorescence detection. Post-ECD oxidation of BH2 or an analog thereof using a conditioning guard cell at the optimum potential oxidizes BH2 or an analog thereof to B or the corresponding biopterin analog. This is desirable because BH2 is not fluorescently active or easily measured and must be converted to B, which is easily measured using fluorescence. Endogenous BH2, once converted to B, and endogenous B are distinguished from one another by two separate fluorescent peaks, due to the different retention times on the HPLC column for each molecule.

In total the methods can be used to measure the species BH4, BH2, and B, and analogs thereof. The biopterins preferably are measured using a 2% MeOH-containing mobile phase, as described herein. Biopterin analogs, such as valine biopterin derivatives, may be better suited to higher methanol contents in the mobile phase, e.g. a 10% MeOH-containing mobile phase.

Thus, a method for detecting biopterins in a mixture of biopterin species can include (a) separating biopterin species in the mixture by reverse phase HPLC; and in the case of BH4 and analogs thereof, (b1) performing electrochemical detection by oxidizing the BH4 and analogs thereof present by a first electrode to quinonoid dihydrobiopterin forms, followed by reducing the quinonoid forms back to BH4 and analogs thereof present at a second electrode, and measuring current generated by the reduction reaction to determine the concentration of species; and/or (b2) in the case of BH2, analogs thereof, biopterin, or analogs thereof, measuring such species by fluorescence detection following post-column oxidation of BH2 species to biopterin. Preferably, the mobile phase is one disclosed herein.

In one embodiment, the preferred mobile phase includes sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol (DTE) with methanol. Preferred concentrations are 50 mM sodium acetate, 5 mM citric acid, 48 μM EDTA, and 160 μM DTE with 2% methanol.

#### VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be

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made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## Example 1

Concentration Time Curve for Biopterin in Plasma  
after a Single Oral Dose in the Rat

The purpose of this study was to assess the pharmacokinetics of BH4 after a single oral administration in rats. Single doses of BH4 (10 and 100 mg/kg) were administered orally to male Sprague Dawley rats (6 weeks old) under fasting conditions.

## Results

The maximum total biopterin concentrations in plasma 2 hrs and 1 hr post-dosing were 108 ng/ml (i.e., about 3× the endogenous level) and 1227 ng/ml (i.e., about 30× the endogenous level), respectively (FIG. 18). Thereafter, biopterin had an elimination half-life ( $t_{1/2}$ ) of about 1.1 hr, returning to the endogenous level 9 hrs post-dosing for the 10 mg/kg dose and 24 hrs post-dosing for the 100 mg/kg dose (FIG. 18).

The bioavailability (F) after a 10 and 100 mg/kg oral administration were 6.8% and 11.8%, respectively, based on the area under the plasma concentration-time curve (AUC) obtained by subtracting the endogenous level during a 10 mg/kg intravenous administration. Rate of GI absorption were 8.8% when measured using radioactive markers in urine. An estimate of the actual value would be approximately 10% oral bioavailability based on these data.

The ratio of reduced biopterin to total biopterins in plasma (i.e., the reduced-form ratio) was relatively static (73%-96%) (FIG. 19).

## Example 2

Concentration Time Curve for Biopterin in Plasma  
after Single Oral Dose to Monkey

The purpose of this study was to assess pharmacokinetics of sapropterin after a single oral administration in cynomolgus monkeys. A single dose of sapropterin (10 mg/kg) was administered orally to female cynomolgus monkeys (3/group) under fasting conditions.

## Results

The total plasma biopterin concentration (ΔC) reached its maximum value 3 hrs post-dosing (344 ng/ml, approximately 20× endogenous levels) (FIG. 20). The plasma elimination half-life of biopterin was approximately 1.4 hrs, returning to the endogenous level within 24 hrs post-dosing. The ratio of reduced biopterin to total biopterins was nearly constant during the test period. The bioavailability (F) following a 10 mg/kg oral administration to female monkeys was about 9%, measured as ΔAUC oral/iv ratios (FIG. 21).

## Example 3

Relative Bioavailability of Tetrahydrobiopterin  
(BH4) Administered after Dissolution of Tablet(s) in  
Water or Administered as Intact Tablet(s), and Effect  
of Food on Absorption in Healthy Subjects

## Objectives

The primary objectives of the study were: (1) to evaluate the relative bioavailability of tetrahydrobiopterin (BH4,

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sapropterin dihydrochloride) when administered after dissolution of tablet(s) in water or administered as intact tablet(s); (2) to compare the effect of food on the bioavailability of BH4 in healthy subjects. The secondary objective of the study was to assess the safety and tolerability of single oral doses of BH4 in healthy subjects.

## Methodology

This study was an open-label, randomized, three-treatment, six-sequence, three-period crossover study in which 30 subjects were to complete 3 single-dose dosing periods and were randomized to one of six sequence groups (Groups 1, 2, 3, 4, 5, and 6):

Group 1: a, b, c

Group 2: b, c, a

Group 3: c, a, b

Group 4: a, c, b

Group 5: b, a, c

Group 6: c, b, a

where all dosing groups received BH4 10 mg/kg orally as follows:

a: administered after dissolution of tablet(s) in water given in fasting under fasting conditions

b: administered as intact tablet(s) given in fasting under fasting conditions

c: administered as intact tablet(s) given 30 minutes after beginning to ingest a high-calorie, high-fat meal in fed conditions

Each subject received a single dose of 10 mg/kg of BH4 during each treatment period. A washout period of at least seven days separated each dose administration. A post-study assessment was performed 5-7 days after discharge of the third treatment period. Blood samples for Pharmacokinetic (PK) analysis were drawn at scheduled collection times during each study period: within 30 minutes prior to dose, and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post-dose.

## Dose and Mode of Administration

BH4 tablets were administered as 10 mg/kg dosages per treatment period. Tablets were administered by a) dissolution in water given in a fasted state, b) as intact tablets given in a fasted state, or c) as intact tablets given in a fed state.

Each dose of study drug was prepared and administered in liquid (solution) form mixed with water. The water supplied was ambient temperature tap water. Dosing solutions were prepared within 15 minutes of scheduled dose time. Dissolution of the tablet in liquid took approximately 1 to 3 minutes. The tablets were broken up or crushed in the dosing cup prior to dissolution in order to improve dissolution rate.

At the designated morning dosing time, BH4 was administered orally as the number of tablets equivalent to a 10 mg/kg dose, dissolved in 120 mL of water or orange juice. Each subject was observed closely as the entire 120 mL dose was consumed within 15 minutes of preparation. Immediately after the dose had been consumed, the dosing cup was rinsed with 60 mL of water and the subject consumed the rinse. A second 60 mL water rinse was added to the dosing cup and then the subject consumed the second rinse. The entire dosing procedure was completed in a 1-minute time period. A qualified staff person inspected the dosing cup and each subject's mouth immediately after completion of the dose to ensure that the entire dose was consumed. Alternatively, the subject swallowed a pill containing the BH4 rather than dissolving it in water. For each individual, the dosing periods occurred with a minimum of 7 days between doses.



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## Food Intake Schedule

A snack was served the evening of check-in. All subjects were then required to fast for at least 10 hours prior to dosing.

## Fasting Conditions

Subjects receiving treatments administered under fasting conditions were dosed after they completed a minimum 10-hour overnight fast.

The subjects continued to fast for 4 hours post dose. Water was allowed ad lib during the study except for 1 hour prior through 1 hour post-dose. Standardized meals were provided at approximately 4 and 10 hours after drug administration and at appropriate times thereafter.

## Non-Fasting Conditions

Subjects receiving treatments administered under non-fasting conditions were dosed after consuming a high-calorie, high-fat breakfast meal. Subjects received the following standard high-fat (approximately 50% of total caloric content of the meal), high-calorie (approximately 1000 calories) breakfast that began 30 minutes prior to scheduled administration of the dose and ended (last bite taken) within 5 minutes prior to dosing.

- 2 eggs fried in butter
- 2 strips of bacon
- 2 slices of toast with butter
- 4 ounces of hash brown potatoes
- 8 ounces of whole milk

This meal contained approximately 150 protein calories, 250 carbohydrate calories, and 500-600 fat calories. An equivalent meal was substituted with documentation of the menu and caloric contents.

The subjects then fasted for 4 hours post dose. Water was allowed ad lib during the study except for 1 hour prior through 1 hour post-dose. Standard meals were provided at approximately 4 and 10 hours after drug administration and at appropriate times thereafter.

## Duration of Treatment

Three single-dose treatment periods were each separated by a minimum of 7 days.

A follow-up visit was conducted 5 to 7 days after the last treatment visit.

## Safety Variables: Evaluation and Methods

Safety was evaluated for all subjects who take at least one dose of BH4.

## Efficacy and Safety Measurements Assessed and Flow Chart

Safety was evaluated by recording the incidence of adverse events, changes in 12-lead ECG parameters, vital signs and physical examination results, and changes in baseline in laboratory test values. The schedule for these assessments is shown in FIG. 22.

## Physical Examinations and Vital Signs

Each subject underwent a routine physical examination by the study investigator. The physical examination included evaluation of head, eyes, ears, nose, throat, neck, heart, chest, lungs, abdomen, extremities, peripheral pulses, neurologic status, skin, and other physical conditions of note are evaluated. This study protocol did not require genitourinary examinations.

Height (in centimeters) and weight (in kilograms) were measured and body mass index (BMI) was calculated ( $BMI = \text{weight (kg)} / [\text{height (m)}]^2$ ).

Blood pressure was measured in the sitting position according to the American Heart Association recommenda-

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tions. Subjects were at rest with their feet on the floor for 5 minutes in the sitting position when blood pressure was measured.

Heart (pulse) rate was measured while the subject was in the sitting position.

A standardized 12-lead electrocardiogram (ECG) recording was taken at screening and at study discharge. ECGs were evaluated by a qualified investigator. Copies of the ECG and evaluation reports were kept as part of each subject's file.

The medical history, clinical laboratory test results and ECG tracing(s) were reviewed and evaluated by the Principal Investigator to determine clinical eligibility of each subject to participate in the study.

## Clinical Laboratory Assessments

## Hematology:

The following were evaluated: hemoglobin, hematocrit, total and differential leukocyte count, red blood cell (RBC) and platelet count.

In addition, blood was tested for Hepatitis B Surface Antigen, Hepatitis C Antibody and Human Immunodeficiency Virus (HIV).

## Chemistry:

The following were evaluated: albumin, blood urea nitrogen (BUN), creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ), lactic dehydrogenase (LDH), uric acid, and glucose.

## Urinalysis:

The following were evaluated by the urine dipstick method: pH, specific gravity, protein, glucose, ketones, bilirubin, blood, nitrite, and urobilinogen. If protein, occult blood, or nitrite values are out of range, a microscopic examination is performed.

Urine samples were also tested for drugs of abuse (amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine and opiates).

## Adverse Events

In this study, an adverse event (AE) was defined as any untoward medical occurrence in a subject or clinical investigation subject administered BH4, at any dose, whether or not it has a causal relationship with the event. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of BH4, whether or not related to BH4. This definition included intercurrent illnesses or injuries and exacerbation (increase in frequency, severity or specificity) of pre-existing conditions.

The reporting period for AEs began with the first administration of BH4. The reporting period for serious adverse events (SAEs) began earlier, from the time of the signing of the Informed Consent. SAEs were defined later in this section. The investigator monitored all AEs until resolution or, if the AE was determined to be chronic, a cause was identified. If an AE remained unresolved at the conclusion of the study, the PI and Medical Monitor made a clinical assessment as to whether continued follow-up of the AE was warranted, and documented the results. Assessment of severity was one of the responsibilities of the investigator in the evaluation of AEs and SAEs. The investigator was responsible for applying his or her clinical judgment to assess the causal relationship of each AE to BH4.



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## Serious Adverse Events

A serious adverse event (SAE) was defined as any AE that has at least one of the following outcomes:

Resulted in death

Was life-threatening, that is, placed the subjects at immediate risk of death from the event as it occurred

This definition did not include a reaction that, had it occurred in a more severe form, might cause death

Required inpatient hospitalization or prolongation of existing hospitalization

Admission of a subject to the hospital as an inpatient as a result of an AE, even if the subject was released on the same day, qualified as hospitalization. An emergency room visit did not constitute hospitalization.

Resulted in persistent or significant disability or incapacity

An event qualified as resulting in a persistent or significant disability or incapacity if it involved a substantial disruption of the subject's ability to carry out usual life functions. This definition was not intended to include experiences of relatively minor or temporary medical significance.

Was a congenital anomaly or birth defect, that is, an AE that occurred in the child or fetus of subject exposed to study drug prior to conception or during pregnancy

Was an important medical event that did not meet any of the above criteria, but could jeopardize the subject or required medical or surgical intervention to prevent one of the outcomes listed above.

More than one of the above outcomes could apply to any specific event.

## Appropriateness of Measurements

The measures of safety in this study were routine physical examinations, vital signs, adverse event incidence and severity, and clinical and laboratory procedures.

## Drug Concentration Measurements

Blood (plasma) pharmacokinetic (PK) characteristics were assessed after each dose of study medication. All subjects remained seated in an upright position for 4 hours post-dose. The blood samples were drawn within 30 minutes prior to dose and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post dose. Samples were collected in appropriately labeled 6 mL K<sub>2</sub>-EDTA purple top Vacutainer® tubes. Blood samples were centrifuged at approximately 3000 rpm at 4° C. for 10 minutes. From the resulting plasma, exactly 1 mL was removed from each sample using a pipet, and placed into an aliquot tube containing 0.1% w/v dithioerythritol. The sample was capped and vortexed for approximately 10 seconds using a VWR Mini Vortexer at speed 6. After completion of these steps, the sample was flash frozen in an isopropyl/dry ice bath and placed in a -70° C. freezer pending analysis.

Approximately 80 mL of blood was drawn during each treatment period (5 mL per timepoint) for the PK analysis.

## Pharmacokinetics:

Pharmacokinetic (PK) analysis of plasma BH4 concentration-time data was performed using non-compartmental methods to obtain estimates of the following PK parameters:

Peak plasma concentration ( $C_{max}$ ) and time to peak concentration ( $T_{max}$ ), obtained directly from the data without interpolation;

$\lambda_z$ , the apparent terminal elimination rate constant, determined by log-linear regression of the terminal plasma concentrations;

Area under the plasma concentration-time curve from time zero to the time of last measurable concentration [AUC (0-t)], calculated by the linear trapezoidal method;

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The apparent elimination half-life ( $t_{1/2}$ ), calculated as  $0.693/\lambda_z$ ;

Area under the plasma concentration-time curve from time 0 to infinity [AUC(inf)] where  $AUC(inf) = AUC(0-t) + C_t/\lambda_z$  and  $C_t$  is the last measurable concentration.

## Estimation of Absorption Rate

Subjects were given a 10 mg/kg oral or intravenous dose of BH4, followed by serial measurements of plasma total bioppterin concentration to determine the rate of BH4 absorption from the gastrointestinal tract from the area under the plasma total bioppterin concentration increase ( $\Delta C_p$ )-time curve ( $\Delta AUC$ ). It was anticipated that a lower dose of BH4 was required when administered intravenously in comparison with BH4 administered orally to achieve the same level of bioavailability. For example, it may require 10 mg/kg of BH4 given orally to achieve the same level of bioavailability as 1 mg/kg BH4 administered intravenously. Because the manner of administration enhanced bioavailability, it may require only 5 mg/kg of BH4 to achieve the same level of bioavailability as a 1 mg/kg IV dose of BH4.

The rate of BH4 absorption from the gastrointestinal tract was estimated from the area under the plasma total bioppterin concentration increase ( $\Delta C_p$ )-time curve ( $\Delta AUC$ ) after the administration BH4 using the following formulas:

## Estimation from AUC

$$\text{Absorption rate (\%)} = (\Delta AUC \text{ after p.o. dose} / \Delta AUC \text{ after i.v. dose}) \times (\text{i.v. dose} / \text{p.o. dose} \times 100)$$

## Statistical Methods:

Comparison of the pharmacokinetic parameters  $C_{max}$ , AUC(0-t), and AUC(inf) for BH4 was conducted using an analysis of variance (ANOVA) model with sequence, subject within sequence, treatment, and period as the classification variables using the natural logarithms of the parameters as the dependent variables. The comparisons of interest were between the dissolved and intact tablet in the fasted state and the intact tablet in the fed and fasted states.

The data from all subjects completing at least two study periods were included in the PK statistical analyses. All subjects receiving at least one dose of study drug were included in the safety analyses.

All PK and associated statistical analyses were done using SAS® for Windows® Version 9.1.3 or higher.

To provide sufficient power to meet the objectives of the study, a sample size of approximately 30 subjects, each with 3 treatment periods, was considered adequate to provide estimates of the differences comparisons of interest. No formal sample size calculation was conducted.

## Results

## Pharmacokinetics

## Intact Versus Dissolved Tablets

Mean plasma concentrations of BH<sub>4</sub> were lower when BH4 was administered as a dissolved tablet compared to the intact tablet (FIGS. 23 and 24). Mean  $C_{max}$  was higher for the intact tablet as were mean values for AUC(0-t) and AUC(inf) (FIG. 25). The geometric mean ratios, intact-to-dissolved tablet, ranged from 118% to 121% and the upper limits of the associated 90% confidence intervals were greater than 125% (FIG. 26), indicating a statistically significant increase in absorption when the intact tablet is administered with a high-calorie, high-fat meal difference in absorption between dissolved and intact tablet administration. The median and range for  $T_{max}$  were essentially the same for the dissolved and intact tablets (FIG. 25), suggesting that the increase seen with the intact tablet was in the extent but not the rate of absorption.

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## Effect of High-Calorie, High-Fat Food on Drug Absorption

As expected, administration of the intact tablet with a standard high-fat high-calorie meal resulted in a substantial increase in the mean plasma BH<sub>4</sub> concentrations (FIG. 23) and mean values for C<sub>max</sub>, AUC(0-t), and AUC(inf) (FIG. 25). The geometric mean ratios (fed-to-fasted) ranged from 126% to 139% (FIG. 26) and, consequently, the upper limits of the associated 90% confidence intervals were greater than 125%, indicating a statistically significant difference in the effect of food on absorption compared to intact tablets. The median and range for T<sub>max</sub> were essentially the same under fed and fasted conditions (FIG. 25), suggesting that the increase seen with food was in the extent but not the rate of absorption of absorption.

## Safety:

There were no serious adverse events (SAEs) in this study. Five (5) subjects reported a total of 9 adverse events (AE)s. Eight (8) of these 9 AEs were assessed as mild and 1 was assessed as moderate in severity. The most common AE was headache; 1 subject experienced a moderate headache which was assessed as unrelated to the study drug, and one subject experienced mild headache on two occasions, both of which were assessed as possibly related. In all, five events were judged to be unrelated and 4 were judged to be possibly related to the study drug. Study exit assessments, ECG and physical examination evaluations were completed with no clinically significant findings.

## Conclusions:

Administration of BH<sub>4</sub> as an intact tablet resulted in an approximate 20% increase in the extent of absorption compared to a dissolved tablet.

Administration of BH<sub>4</sub> as an intact tablet with a high-calorie, high-fat meal under fed conditions resulted in an approximate 30% increase in the extent of absorption compared to fasted conditions.

No clinically significant issues and safety parameters safety issues were identified in this study population. There were no AEs considered serious in this study. Among the 9 AEs reported, all but one, an instance of headache, was mild, and it was assessed to be unrelated to the study drug. Instances of fatigue and headache were the only AEs which were possibly related to the study drug, but and these were assessed as mild in severity.

## Example 4

Formulation Approaches to Enhance Bioavailability of BH<sub>4</sub>

Two control formulations (BH<sub>4</sub> intravenous formulation and BH<sub>4</sub> tablet for oral solution) and six test formulations were selected for testing in animal studies. Each formulation prototype contained 80 mg or 100 mg of BH<sub>4</sub>.

BH<sub>4</sub> Intravenous Formulations

Table 3 lists the composition of an intravenous formulation. BH<sub>4</sub> was passed through a #20 mesh stainless screen before use while mannitol was used as received. This formulation was filled as a powder in a bottle and constituted with sterile water for injection prior to administration. Each bottle contained 100 mg of BH<sub>4</sub> and 5 g of mannitol in a clear polyethylene terephthalate copolyester (PETG) bottle with a white high-density polyethylene (HDPE) screw top closure. Prior to administration, the formulation was constituted with 100 mL of sterile water for injection to yield a final concentration of 1 mg/mL. The IV formulation was supplied as a dry

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powder in a bottle, and each bottle contained the API and mannitol. The powder was dissolved in sterile water for injection and filtered prior to administration by IV route.

TABLE 3

Composition of BH <sub>4</sub> IV Formulation		
Ingredients	% (w/v)	mg/mL
BH <sub>4</sub>	0.1	1.0
Mannitol (low in endotoxin), USP/Ph.Eur.	5.0	50.0
Sterile water for injection	qs 100 mL	qs 1 mL

BH<sub>4</sub> Tablet for Oral Solution

Table 4 lists the composition of an oral solution formulation. Ten (10) BH<sub>4</sub> tablets (100 mg) were placed into a 125 mL graduated PETG bottle with a white HDPE closure. Prior to administration, the formulation was constituted with 100 mL of sterile water for injection to yield a final concentration of 10 mg/mL.

TABLE 4

Composition of BH <sub>4</sub> Tablet, 100 mg		
Ingredients	% (w/w)	mg/tablet
BH <sub>4</sub>	33.33	99.99
Ascorbic Acid, USP/EP	1.67	5.01
Crospovidone, USP/EP	4.5	13.5
Dicalcium Phosphate Anhydrous, USP/EP	2.18	6.54
Mannitol (Parteck M 200), UPS/EP	57.06	171.18
Riboflavin universal, USP/EP	0.01	0.03
Sodium Stearyl Fumarate (PRUV), NF/EP	1.25	3.75
Total	100.00	300.00

## Formulation Prototype to Slow Gastro-Intestinal Motility

Table 5 lists the composition of a delayed gastric emptying time prototype. BH<sub>4</sub> was passed through a #20 mesh stainless steel screen before use. The Capmul GMO-50 was melted in a 37° C. water bath. BH<sub>4</sub> and ascorbic acid were weighed and added slowly to the melted Capmul while stirring vigorously. The solid dispersion was added dropwise into a size #2 capsule using a pipette. Three filled capsules were placed in a 100 cc high-density polyethylene (HDPE) bottle with a heat-induction seal closure.

TABLE 5

Composition of BH <sub>4</sub> Delayed Gastric Emptying Time Oral Capsule Formulation		
Ingredients	% (w/w)	mg/capsule
BH <sub>4</sub>	25	80
Glyceryl mono/di-oleate (Capmul GMO-50)	65	208
Ascorbic acid fine powder	10	32
Total	100	320

## Bioadhesive Prototype

Table 6 lists the composition of a bioadhesive prototype. All materials, except for Carbopol 71G, were passed through a #20 mesh stainless steel screen. All materials were weighed

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and added to a plastic bag having a zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. The powder was compressed into a tablet using a 1/4" standard, round, concaved, plain-faced B tooling on a Globe Pharma MTCM-I manual press at 600 psi. Three tablets along with a silica gel desiccant canister were packaged in a 100 cc HDPE with a heat-induction seal closure.

TABLE 6

Composition of BH4 Bioadhesive Oral Tablet Formulation		
Ingredients	% (w/w)	mg/tablet
BH4	48.5	80.00
Carbopol 71 G	20.0	32.99
Polycarbophil (Noveon AA1)	20.0	32.99
Ascorbic acid fine powder	10.0	16.49
Sodium stearyl fumarate (PRUV)	1.5	2.47
Total	100.0	164.94

## Sustained Release Prototype

Table 7 lists the composition of a sustained release prototype tested in the monkey. All materials, except for Methocel K100M Premium CR, were passed through a #20 mesh stainless steel screen. All materials were weighed and added to a plastic bag having zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. The powder was compressed into a tablet using a 1/4" standard, round, concaved, plain-faced B tooling on a Globe Pharma MTCM-I manual press at 1200 psi. The tablets along with a Silica gel desiccant canister were packaged in a 100 HDPE bottle with heat-induction seal closure.

TABLE 7

Composition of BH4 Sustained Release Tablet Formulation		
Ingredients	% (w/w)	mg/tablet
BH4	53.5	80.00
Methocel K100M premium CR	35.0	52.34
Ascorbic acid fine powder	10.0	14.95
Sodium stearyl fumarate (PRUV)	1.5	2.24
Total	100.0	149.53

## Proton Donor Polymer Prototype

Table 8 lists the composition of a proton donor polymer prototype tested in the monkey. All materials, except for Eudragit L100-55 and Kollidon CL, were pre-screened using a #20 mesh stainless steel screen. All materials were weighed and added to a plastic bag having a zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. A pre-weighed quantity of powder was filled into a size #2 capsule.

A coating solution was prepared by dissolving Eudragit L100-55 and Carbowax PEG 4600 in Ethyl Alcohol. The Eudragit L100-55 and Carbowax PEG 4600 were weighed and added to a 125 mL graduated polyethylene terephthalate copolyester bottle (PETG). The Ethyl Alcohol was added to the PETG bottle, and it was placed in a 40° C. water bath with sonication until the solution was clear.

The powder-filled capsules were manually dipped into the coating solution and allowed to dry at 40° C. for 20 minutes. The dried capsules were weighed and then rolled in Sylloid FP244 to remove residual tackiness. Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

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TABLE 8

Composition of BH4 Proton Donor Capsule Formulation		
Ingredients	% (w/w)	mg/capsule
BH4	40.0	80
Eudragit L100-55	44.5	89
Crospovidone (Kollidon CL)	4.0	8
Ascorbic acid fine powder	10.0	20
Sodium stearyl fumarate (PRUV)	1.5	3
Total	100.0	200
Ingredients	% (w/w)	mg/capsule <sup>1</sup>
Eudragit L100-55	5.0	ND
Polyethylene glycol 4600 (Carbowax Sentry)	5.0	ND
Ethyl alcohol, 200 proof	100 mL	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains about 1 to 3% weight in polymer coating.  
ND = Not Determined

## Floating Delivery System

Table 9 lists the composition of a floating delivery system. All materials, except for Eudragit L100-55, were passed through a #20 mesh stainless steel screen. This tablet prototype comprised three layers; the middle layer contained the drug substance, which was sandwiched between two water-insoluble outer layers. The inner and outer materials were weighed and added separately to plastic bags having zip-locking closures, which were then shaken until the mixtures appeared uniform.

The two outer layers (12 mg each) and inner layer (14.5 mg) were weighed. One of the outer layers was added to the press, followed by the inner layer, and then the last outer layer. The layers were compressed into a tablet using a 3/16" round, beveled, plain-faced B Tooling on a Globe Pharma MTCM-I manual press at 200 psi.

A coating solution was prepared by dissolving Ethocel and PEG 4600 in an ethyl alcohol and purified water mixture. The ingredients were added to a PETG bottle, which was mixed and placed in a 40° C. water bath with sonication until the solution appeared clear.

The tablets were manually dipped in the coating solution and allowed to dry for 20 minutes at 40° C. Each tablet was re-weighed after coating. Seven (7) tablets were placed into each of the size #2 elongated capsules. Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

TABLE 9

Composition of BH4 Floating Dosage Formulation		
Ingredients	% (w/w)	mg/tablet
Outer Layers 1 and 3		
Eudragit L100-55	49.5	5.94
Stearic acid	49.5	5.94
Sodium stearyl fumarate (PRUV)	1.0	0.12
Total	100.0	12.00
Ingredients Middle Layer 2		
BH4	79.0	11.46
Stearic acid	10.0	1.45

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TABLE 9-continued

Composition of BH4 Floating Dosage Formulation		
Ascorbic acid fine powder	10.0	1.45
Sodium stearyl fumarate	1.0	0.15
Total	100.0	14.51
Ingredients		
7 tablets in a Capsule	% (w/w)	mg/capsule
BH4	29.8	80.19
Stearic acid	34.6	93.31
Ascorbic acid fine powder	3.8	10.15
Eudragit L100-55	30.8	83.16
Sodium stearyl fumarate (PRUV)	1.0	2.70
Total	100.0	269.51
Ingredients		
Tablet Coating Solution	% (w/w)	mg/capsule <sup>1</sup>
Ethocel Standard 10 FP	5.0	ND
Carbowax PEG 4600	5.0	ND
Ethanol 200 proof	95.0 mL	ND
Purified Water	5.0 mL	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains about 3 to 8% weight in polymer coating.  
ND = Not Determined

## Gas Generating Floating Delivery System

Table 10. lists the composition of a gas generating floating delivery system. This formulation was composed of a core tablet containing the drug substance surrounded by a gas-generating outer layer. All materials, except for sodium bicarbonate and Methocel K100M CR, were pre-screened using a #20 mesh stainless steel screen. The inner core and outer layer materials were weighed and added separately to plastic bags having zip-locking closures, which were closed and shaken until the mixture appeared uniform. The blended powder for the inner core (35 mg) was compressed into a tablet using a 1/8" round, beveled, plain faced B Tooling on a Globe Pharma MTCM-I manual press at 800 psi.

A coating solution was prepared by dissolving using Ethocel and PEG 4600 in ethyl alcohol. The inner core tablets were manually dipped in the coating solution and allowed to dry for 20 minutes at 40° C. The blended powder for the outer layer (40 mg) was weighed. One half was added to the press, followed by the inner core tablet, and then the second half of the outer layer. The tablet was compressed using a 3/16" round, beveled, plain-faced B Tooling on a Globe Pharma MTCM-I manual press at 800 psi. Four (4) tablets were placed into each size #2 capsule.

TABLE 10

Composition of BH4 Gas Generating Floating Dosage Formulation		
	% (w/w)	mg/tablet
Ingredients		
Inner tablet Core		
BH4	58.3	20.39
Ascorbic acid fine powder	19.4	6.80
HPMC K100MCR	19.4	6.80
Sodium stearyl fumarate (PRUV)	2.9	1.02
Total	100	35.01

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TABLE 10-continued

Composition of BH4 Gas Generating Floating Dosage Formulation		
Ingredients		
Outer Tablet Layer		
HPMC K100MCR	46.1	18.46
Citric acid anhydrous	34.2	13.68
Sodium bicarbonate	17.1	6.84
Sodium stearyl fumarate	2.6	1.03
Total	100	40.01
Ingredients		
Four tablets in a Capsule	% (w/w)	mg/capsule
BH4	27.2	81.55
Ascorbic acid fine powder	9.1	27.18
HPMC K100MCR	33.7	101.03
Citric acid anhydrous	18.2	54.70
Sodium bicarbonate	9.1	27.35
Sodium stearyl fumarate	2.7	8.18
Total	100	299.99
Ingredients		
Coating Solution	% (w/w)	mg/capsule <sup>1</sup>
Ethocel Standard 10 FP	5.0	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains weight in polymer coating.  
ND = Not Determined

## Bioadhesive Granule Prototype

Table 11 lists the composition of a bioadhesive granule prototype. All materials, except for Methocel K100M CR, were pre-screened using a #20-mesh stainless steel screen. All materials, except for the sodium stearyl fumarate (PRUV), were weighed and placed into a size #1 granulator bowl (LB Bohle Mini Granulator BMG). The powder was mixed at an impeller speed of 300 rpm and a chopper speed of 2500 rpm for five minutes until the mixture appeared uniform. Maintaining the impeller and chopper speeds, 5 mL of ethyl alcohol was added dropwise to the mixture until granules formed. The wet mass was removed from the granulation bowl and screened through an 18-mesh stainless steel screen. The granules were collected and placed in a 40° C. oven to dry for one hour. The loss on drying of the granules was determined to be 1.93% after one hour of drying. The granules were weighed and placed into a plastic bag having a zip-locking closure. Sodium stearyl fumarate (PRUV) was added to the dried granules in the bag. The bag was closed and shaken until the sodium stearyl fumarate (PRUV) appeared evenly distributed among the granules. The granules were weighed (134 mg). Size 2 elongated capsules were filled with portions of the granules alternating with drops of partially hydrogenated vegetable oil (350 µL). Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

TABLE 11

Composition of BH4 Bioadhesive Granule Capsule Formulation		
Ingredients	% (w/w)	mg/capsule
BH4	60	80.00
Methocel K100M CR	19	25.33
Carbopol 971	10	13.33
Ascorbic Acid fine power	10	13.33



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TABLE 11-continued

Composition of BH4 Bioadhesive Granule Capsule Formulation		
Ingredients	%(w/w)	mg/capsule
Sodium Stearyl Fumarate (PRUV)	1	1.33
Pureco HSC-1 oil	—	350 $\mu$ L
Total	100	133.33

## In Vitro Drug Release

In vitro drug release testing from tablets was conducted according to the USP 27 apparatus II specifications using a Distek 2100C Dissolution Tester (Distek, Inc., North Brunswick, N.J.), along with an Agilent UV-Visible spectroscopy system (Agilent Technologies, Santa Clara, Calif.). The dissolution medium used for the release testing of BH4 was 900 mL of 0.1N HCl. During dissolution testing, the media in each vessel was maintained at  $37^{\circ}\pm 0.5^{\circ}$  C. and agitated at 50 rpm. A sample volume of 5 mL was taken at pre-determined time points. To determine the concentration of BH4 in the samples, 250  $\mu$ L of each sample was diluted with 500  $\mu$ L of 0.1N HCl and the absorption was measured at 265 nm using a UV spectrometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, Santa Clara, Calif.). The data were collected using ChemStation software (Rev. A.09.01[76], Agilent Technologies, Santa Clara, Calif.). All dissolution tests were performed in triplicate.

## Tablet Buoyancy Testing

The buoyancy of the floating prototype tablets was first determined by placing the tablets in plastic cups with 25-50 mL of 0.1N HCl. This test determined the time necessary for the tablets to float as well as the duration of their floating with no agitation. Those prototypes that floated for at least four hours were submitted for dissolution testing. During the dissolution testing, the buoyancy of the tablets was determined using the paddle method at a rotation speed of 50 rpm. The state of the tablets was checked visually at various time points.

## Disintegration Testing

Disintegration testing was conducted according to the USP-27 disintegration test specifications using a Distek 3100 Series Disintegration Tester (Distek Inc., North Brunswick, N.J.). The disintegration media used was 900 mL of 0.1N HCl or 900 mL of 0.2M Potassium Phosphate pH 5.8. During the disintegration testing the media in the vessels was maintained at  $37^{\circ}\pm 0.5^{\circ}$  C. The tablets and capsules were visually inspected for disintegration.

## Tablet Hardness Testing

Tablet hardness was determined using a Dr. Schleuniger Pharmatron 8M Tablet Hardness Tester (Dr. Schleuniger® Pharmatron Inc., Manchester, N.H.). The tablets were placed into the jaw of the hardness tester, and the hardness was measured in kiloponds (Kp).

## Tablet Thickness

The thickness of the tablets was measured using a Mitutoyo Digimatic Indicator (Mitutoyo Absolute, Dr. Schleuniger Pharmatron Inc., Manchester, N.H.). The tablets were placed under the thickness gauge and the value indicated was recorded in millimeters (mm).

## Results and Discussion

Several prototypes were developed based on three concepts: gastroretentive, proton donor polymer to change intes-

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tinal pH, and sustained release dosage forms. The sections below described the formulation development of each prototype.

BH4 Intravenous Formulation—After sterile water constitution, the resulting solution was isotonic, pH 3.2 and contained 1 mg/mL of BH4, and was suitable for intravenous administration after sterile filtration through a 0.22 micron filter. Stability of the 1 mg/mL solution stored at ambient temperature was analyzed by HPLC every hour for three hours. The aged solution samples were then stored at  $-20^{\circ}$  C. and analyzed by HPLC after 2 weeks. FIG. 27 indicates that the solution was stable at ambient temperature for at least 3 hours after constitution and was stable for at least 2 weeks during storage at  $-20^{\circ}$  C.

## BH4 Tablet for Oral Solution

Each bottle was packaged to contain ten (10) BH4 tablets, 100 mg. One-hundred (100) mL of purified water or sterile water for injection was added to the contents of each bottle. Following vigorous shaking of the bottle, the tablets rapidly disintegrated within 5 minutes. The resulting solution contained 10 mg/mL of BH4 for oral administration. Not all the ingredients in the tablet were soluble, and although the final solution appeared hazy or translucent, the active pharmaceutical ingredient was fully dissolved and the fine particulates were poorly soluble inactive ingredients.

## Formulation Prototype to Slow Gastro-Intestinal Motility

This capsule formulation comprised of BH4 and ascorbic acid dispersed in a semi-solid fatty acid derivative (glyceryl mono/di-oleate, melting point of  $86^{\circ}$  F. ( $30^{\circ}$  C.)). Glyceryl mono/di-oleate (GMO) was also selected because GMO is chemically compatible with BH4. The dissolution profile depicted in FIG. 28 showed that over 90% of the drug was released in 2 hours and the dissolution profile remained unchanged after the capsules were stored at  $40^{\circ}$  C. for 57 days.

The drug dispersion in melted GMO, a semi-solid, was filled into hard gelatin capsules manually. The density of the semi-solid is greater than 1 g/mL, and it was possible to fill at least 80 mg dose at 25% drug loading in a size #2 capsule. It is expected that a size #0 capsule should be able to contain at least 200 mg of drug using the same formulation. Leakage of fatty acid from the capsule was observed during storage at  $40^{\circ}$  C. Preferably, capsules or softgel capsule formulations will be banded to avoid leaking of fatty acid during storage.

## Bioadhesive Prototype

Many bioadhesives are made of either synthetic or natural polymers. Most of the current synthetic bioadhesive polymers are either polyacrylic acid or cellulose derivatives. Examples of polyacrylic acid-based polymers include but are not limited to carbopol, polycarbophil, polyacrylic acid (PAAc), etc. Cellulosics include but are not limited to hydroxypropyl cellulose and hydroxypropylmethyl cellulose (HPMC). Two bioadhesive prototypes were developed for testing in animal studies. The first prototype was a bioadhesive tablet formulation and the second a capsule containing bioadhesive granules.

Polycarbophil and carbomer polymers were selected for the development of the first bioadhesive tablet prototype. Carbopol 71 G is a granular form of carbomer and has good powder flow properties. All the batches of the fabricated tablets were of good quality with acceptable drug content (evident by close to 100% drug release in dissolution profiles) and acceptable hardness. Table 12 lists the representative tablet weight, thickness, and hardness of the bioadhesive prototype containing carbomer and polycarbophil.

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TABLE 12

Representative Tablet Weight, Thickness and Hardness for Bioadhesive Prototype containing Carbomer and Polycarbophil				
Tablet Lot Number	Compression Pressure (psi)	Weight (mg)	Thickness (mm)	Hardness (Kp)
11210-83	600	165.4	5.24	10.5
11229-4	600	166.7	5.64	10.3
11229-4	800	164.1	5.27	14.4
11229-4	1000	164.9	5.12	18

HPMC and carbomer polymers were used for the development of the second bioadhesive granules. HPMC was selected because it is used as low-density hydrocolloid system and controlled drug release independent of pH. Granules were selected over tablet to increase the chance of bioadhesion by increasing the surface area of the dosage form. To facilitate the separation of the granules-filled capsule in dissolution medium, the granules were coated partially with hydrogenated oil. Without the oil coating, the granules hydrated and formed a capsule-shaped matrix without disintegrating into individual granules.

The release profiles of the two bioadhesive prototypes (tablet and granules) are shown in FIG. 29, which shows that the release profile of the tablet was longer than the granules. Drug release was about 90% in four hours and 95% in one hour for the tablet and granules bioadhesive dosage forms, respectively. Upon storage at 40° C. and ambient humidity for one month without moisture protection (no heat induction seal), the tablet prototype exhibited a slowdown in drug dissolution (FIG. 29). For prototypes containing carbomer, moisture protection precaution should be taken to protect the tablet from possibly hydrating prematurely. Sustained Release Prototype

Hydroxypropylmethylcellulose (HPMC) is used as a hydrophilic vehicle for the preparation of oral controlled drug delivery systems (Colombo, *Adv. Drug Deliv. Rev.*, 1993, 11, 37). HPMC matrices are known to control the release of a variety of drugs (Chattaraj, et al. *Drug Develop. Ind. Pharm.*, 1996, 22, 555; Pabon, et al., *Drug Develop. Ind. Pharm.*, 1992, 18, 2163; Lee, et al., *Drug Develop. Ind. Pharm.*, 1999, 25, 493; Basak, et al., *Indian J. Pharm. Sci.*, 2004, 66, 827; Rajabi-Siabhooni, et al., *J. Pharm. Pharmacol.*, 1992, 44, 1062). Various viscosity grades of HPMC (K4M, K15M and K100 M) to control the release of BH4 were evaluated in this study. The dissolution profiles of tablets made with various grades of HPMC are shown in FIG. 30. Drug release profiles were similar at 20% HPMC regardless of viscosity grade; over 80% of the drug was released in 2 hours. When HPMC polymer was exposed to aqueous medium, it underwent rapid hydration and chain relaxation to form gel layer (Naruhashi, et al., *Pharm Res.* 2003, 19:1415-1421). The HPMC at 20% may not form a substantial gel barrier layer to slow the release of BH4 significantly.

The dissolution profiles of tablets produced with varying concentrations (20% to 40%) of a high viscosity grade of HPMC (Methocel K100M CR) are presented in FIG. 30. A tablet containing 35% to 40% Methocel K100M CR was found to slow drug release for up to four hours whereas 20% HPMC released drug in two hours (FIG. 31). A tablet containing 35% HPMC (Methocel K100M) was selected as the prototype for testing in animal studies because it contained the least amount of HPMC required to slow the drug release for up to four hours. As such, the tablets were of good quality with acceptable drug content as evident by close to 100% drug release in dissolution profiles.

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## Proton Donor Polymer Prototype

To increase the oral absorption of BH4, one approach is to stabilize the drug by decreasing the pH of the proximal small intestine. To manipulate intestinal luminal pH, Eudragit L100-55, a proton-releasing polymer commonly used for enteric coating, was selected. This polymer is not soluble under acidic conditions, and it becomes soluble and releases protons under weakly acidic (pH>5.5) to alkaline condition due to its carboxyl groups, thereby controlling the intestinal luminal pH to be acidic. Naruhashi, et al. (2003) found that pH in the lumen was decreased in a Eudragit L100-55 concentration-dependent manner and the absorption of cefadroxil and cefixime from the ileal loop was increased in the presence of the acidic polymer (Nozawa, et al., *J. Pharm Sci.* 2003, 92 (11), 2208-2216). Nozawa, et al (2003) showed that Eudragit decreased the pH in the intestinal loops, and increased the disappearance of both cefadroxil and cefixime from the loops.

Powder formulations containing BH4 and Eudragit L100-55 as shown in Table 8 were compressed into tablets and filled into capsules. The tablet formulation released about 27% drug in one hour in simulated gastric fluid (SGF) during dissolution testing. However, during disintegration testing, the tablet remained intact in SGF and pH 5.8 phosphate buffer (PB) for at least 2 hours. Even in the presence of a superdisintegrant (croscopovidone or croscarmellose), the tablet failed to disintegrate. It is possible that the drug may be acidifying the Eudragit, creating a low micro pH environment such that the polymer remained unionized and insoluble.

The powder filled capsule drug-Eudragit formulation disintegrated rapidly in SGF. To target proton release in the proximal intestine, an enteric coat was applied to the capsule. Following capsule coating and drying in the oven at 40° C., the capsule gained about 1 to 3% weight in polymer coating. When tested using the USP dissolution apparatus II (paddle), dissolution medium 0.1 N HCl maintained at 37° C. at a rotational speed of 50 rpm, the coated capsule released about 25% of drug in one hour. Following 1 hour of acid (0.1 N HCl) pre-treatment, the coated capsule was placed in a USP disintegration Apparatus with 500 mL of pH 5.8 phosphate buffer maintained at 37° C., the coated capsule disintegrated in about 1 hour. The enteric-coated capsule prototype was selected over the tablet or the uncoated capsule because the enteric-coated capsule was more likely to deliver proton-releasing polymer to the target site.

## Floating Delivery System

Two floating delivery systems were developed. The first prototype was a floating multiple unit dosage form; the purpose of this dosage form was to increase the chance that one of the units will remain in the gastric region and hence prolong the gastric residence time of drugs. This dosage form consisted of seven triple layer tablets in a capsule; the middle layer contained the drug substance, which was sandwiched between two water-insoluble outer layers (FIG. 32). The outer layers contained stearic acid, a hydrophobic and water-insoluble fatty acid, which provided the necessary buoyancy to the floating tablet. Each tablet was manually coated with an alcoholic solution of ethylcellulose and polyethylene glycol MW 4600 (PEG). Ethylcellulose formed a water insoluble film around the tablet and PEG, which acted as a pore former, modulated the release rate. The dissolution profiles of tablets coated with ethylcellulose and various concentrations (20% to 40%) of PEG solutions are presented in FIG. 33. It was noted that the coated triple layer tablet achieved close to zero-order release kinetics. As expected, the drug dissolution rate increased as the concentration of PEG increased. The



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tablets floated in simulated gastric medium for at least four hours during dissolution studies. Table 9 shows the composition of the formulation tested in animal studies.

The second prototype was a gas-generating dosage form. It was formulated in such a way that when it came in contact with acidic gastric contents, carbon dioxide was liberated and got entrapped in the swollen hydrocolloids, which provided buoyancy to the dosage form (FIG. 33). This formulation floated in simulated gastric medium for at least four hours during dissolution studies. However, for such a system to work consistently, the tablets have to be produced in a low humidity environment to prevent premature acid and base reaction. There could be potential interaction between BH4 and sodium bicarbonate in the tablet during storage. For these reasons, this dosage form was not tested in animal studies.

Six prototype test formulations that incorporated various formulation approaches including proton donor polymer to decrease intestinal pH, gastroretentive dosage forms, and sustained released formulations, were developed for animal bioavailability studies.

#### Example 5

##### Bioavailability of Novel BH4 Formulations

The objective of this study was to enhance the absorption of BH4 by developing dosage forms that increase the residence time of the drug in the gastrointestinal (GI) tract.

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Methods: Three healthy cynomolgus monkeys weighing 3-4 kg were used in open, 8-period non-crossover study to determine the bioavailability of seven formulations compared to a control dissolved BH4 formulation. After an overnight fast, the monkeys received, on separate occasions, a single dose of 80 mg of the same novel formulation orally or intravenously with an interval of at least a 1 week washout period between the various novel formulations studied. For intravenous administration, blood samples were collected before dosing and then at 5, 15 and 30 min and 1.0, 2.0, 4.0, 6.0, 8.0, 12 and 24 hr post dose. For oral administration, blood samples were taken before dosing and then 15 and 30 min and 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12, and 24 hr following each dose. Following separation of the plasma by centrifugation, 200-μL aliquots of each sample were promptly transferred into individual tubes containing 0.1% DTE and frozen at -70° C. until ready for assay for total L-biopterin.

Study Formulations: The formulations administered are found in Table 13. Three of the formulations were conceptually designed to be gastroretentive via bioadhesive or floating mechanisms to increase GI residence time (carbomer-based, multi-particulate floating granules and bioadhesive granules). Other concepts were based on slowing GI motility to increase residence time of the formulation (glyceryl mono-oleate), reducing the pH of the small intestine and thereby enhancing BH4 chemical stability to enable absorption of intact drug (proton pump) or sustained delivery formulation to ascertain whether it will enhance absorption.

TABLE 13

Phase	Prototype	Dosage Form	Concept	Ingredients
Phase I	IV	IV solution, 1 mg/mL	Control	BH4, D(-)-Mannitol
Phase II	Kuvan Tablets for Solution	Oral Solution, 10 mg/ml	Control	BH4 tablets manufactured by Lyne (Lot# 140651)
Phase III	Glycerol Mono Oleate	Capsule, 80 mg	Slow GI motility	BH4, Capmul GMO-50, Ascorbic Acid
Phase IV	Carbomer Prototype	Tablet, 80 mg	Gastro-retentive, Bioadhesive	BH4, Carbopol 71G, Noveon AA1, Ascorbic Acid, PRUV
Phase V	HPMC prototype	Tablet, 80 mg	Sustained release	BH4, Methocel K100M Premium CR, Ascorbic Acid, PRUV
Phase VI	Eudragit Prototype	Capsule, 80 mg	Proton donor polymer to lower GI pH	BH4, Eudragit L100-55, Ascorbic Acid, Kollidon CL, PRUV, Coating (Eudragit L100-55, Carbowax PEG 4600, Ethyl Alcohol 200 proof)
Phase VII	Multi-floating units	Multiple tablets in capsule, 80 mg	Gastro-retentive, floating	Inner Layer (BH4, Ascorbic Acid, Stearic Acid, PRUV), Outer Layer (Stearic Acid, Eudragit L100-55, PRUV), Coating (Ethocel Standard 10FP, Carbowax PEG 4600, 95% Ethanol)
Phase VIII	Bioadhesive Granules	Granules in capsule, 80 mg	Gastro-retentive, Bioadhesive	Intergranular (BH4, Methocel K100M Premium CR, Carbopol 971, Ascorbic Acid), Extragranular (PRUV, Pureco HSC-1 oil)

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Plasma Assay for Biopterin: BH4 concentrations in plasma were determined by using a validated, specific, reversed-phase LC/MS/MS method. The standard curve was linear over the concentration range of 50 ng/mL to 2500 ng/mL. The lower limit of quantitation for L-biopterin was 50 ng/mL with intraday precision shown by coefficients of variation less than 5%. L-biopterin is stable in frozen monkey plasma stabilized with 0.1% DTE at -70° C. until assayed. BH4 concentrations were calculated from the determined L-biopterin concentrations.

Pharmacokinetic and Statistical Analysis: Pharmacokinetic parameters were determined for plasma BH4 following the administration of the oral and intravenous formulations. The pharmacokinetic parameters are provided in Table 14.

TABLE 14

Phase, Formulation	AUC <sub>last</sub> (ng-hr/mL)	AUC <sub>∞</sub> (ng-hr/mL)	C <sub>max</sub> (ng/mL)	C <sub>last</sub> <sup>a</sup> (ng/mL)	T <sub>max</sub> (hr)	t <sub>1/2</sub> (hr)
2, dissolved tablet	641 (88)	805 (36)	93.6 (31.3)	9.60 (2.20)	2.33 (0.58)	11.7 (2.1)
3, glyceryl mono-oleate	716 (154)	858 (317)	133 (83)	6.47 (3.60)	2.00 (0)	12.1 (10.3)
4, bioadhesive polymer	593 (50.6)	648 (114)	108 (15)	4.46 (3.36)	2.67 (0.58)	6.89 (3.51)
5, sustained release	355 (134)	472 (36)	86.0 (43.1)	12.9 (12.4)	3.33 (0.58)	5.30 (1.73)
6, proton donor	276 (49.8)	282 (49)	68.3 (25.3)	2.97 (0.71)	3.33 (0.58)	1.59 (0.74)
7, floating dosage form	304 (78)	b	59.9 (31.8)	5.90 (0.94)	4.00 (2.00)	b
8, bioadhesive granulations	292 (79)	366 (40.6)	42.5 (12.6)	5.11 (2.43)	3.0 (0)	15.3 (8.2)

## Results

The objective of this study was to identify formulations that enhance the bioavailability BH4 compared to the control dissolved tablet formulation. The mean plasma BH4 concentration-time profiles of the various dosage forms and the control formulation following the oral administration of BH4 are shown in FIG. 35, and the BH4 pharmacokinetic parameters derived from plasma drug concentration-time profiles are given in Table 14. The control formulation (phase 2) is the dissolved tablet.

As shown in FIG. 35, the glyceryl mono-oleate formulation provided the highest AUC<sub>last</sub> and AUC<sub>∞</sub> which are 716 ng-hr/mL and 858 ng-hr/mL respectively. The control dissolved BH4 tablet formulation exhibited AUC<sub>last</sub> and AUC<sub>∞</sub> which are 641 ng-hr/mL and 805 ng-hr/mL respectively (Table 14). The rank order of the formulations from the most to the least bioavailable is: glyceryl mono-oleate>dissolved tablet>bioadhesive polymer tablet>sustained release tablet>floating dosage forms>bioadhesive granulations capsule product>proton donor capsule product.

### Example 6

#### Preparation of Intravenous Formulation of Tetrahydrobiopterin

#### Preformulation Stability Evaluation

In general, the objective of this study was to evaluate the stability of BH4 in buffer solutions ranging in pH from pH 1 to 7 (See Table 15) and in the presence and absence of antioxidants and with or without inert gas in the reaction solutions (See Table 16).

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TABLE 15

Components and Composition of Buffer Solutions to be used for BH4 Preformulation Stability Studies

Components	Quantities
pH 1.2 Buffer (0.1 N HCl)	
Concentrated HCl (12 N)	8.33 mL
Sodium Chloride	2.92 g
Distilled/Deionized Water qs	1000 mL

TABLE 15-continued

Components and Composition of Buffer Solutions to be used for BH4 Preformulation Stability Studies

Components	Quantities
pH 2.1 Buffer (0.01 N HCl)	
pH 1.2 (0.1 N HCl) Buffer	100 mL
Sodium Chloride	7.79 g
Distilled/Deionized Water qs	1000 mL
pH 3 Buffer	
Phosphoric Acid, 15 M, 85%	.347 mL
Sodium Monobasic Phosphate, anhydrous (NaH <sub>2</sub> PO <sub>4</sub> )	6.17 g
Sodium Chloride	6.16 g
Distilled/Deionized Water qs	1000 mL
pH 4 Buffer	
Acetic Acid, Glacial, 100%	2.38 mL
Sodium Acetate, Trihydrate	1.29 g
Sodium Chloride	8.22 g
Distilled/Deionized Water qs	1000 mL
pH 5 Buffer	
Acetic Acid, Glacial, 100%	.87 mL
Sodium Acetate, Trihydrate	4.78 g
Sodium Chloride	6.72 g
Distilled/Deionized Water qs	1000 mL
pH 6 Buffer	
4-Morpholineethanesulfonic (MES) Acid Monohydrate	4.99 g
MES Sodium Salt	5.75 g
Sodium Chloride	7.23 g
Distilled/Deionized Water qs	1000 mL

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TABLE 15-continued

Components and Composition of Buffer Solutions to be used for BH4 Preformulation Stability Studies	
Components	Quantities
<u>pH 7 Buffer</u>	
Sodium Monobasic Phosphate, Monohydrate (NaH <sub>2</sub> PO <sub>4</sub> )	2.56 g
Sodium Dibasic Phosphate, anhydrous (Na <sub>2</sub> HPO <sub>4</sub> )	4.44 g
Sodium Chloride	2.18 g
Distilled/Deionized Water qs	1000 mL

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More specifically, the influence of combining two antioxidants in the presence or absence of inert gas was evaluated at pH 4 to support the formulation of a liquid product, and at a pH 7 to ascertain the contribution of instability at physiologic pH to the low bioavailability of the compound in monkeys and humans (See Tables 17 and 18). The stability of BH4 is expected to be temperature-dependent. Therefore, the compound stability was evaluated at 2-8° C., 25° C., 30° C. and 37° C. to support the determination of predictive long-term shelf lives for the compound at different temperatures. Determination of the stability of the compound at the physiologic temperature of 37° C. provides data to support the estimation of the stability lifetime of a formulated oral dosage form in the absorptive regions of the GI tract.

TABLE 16

Composition of Buffer Solutions for Stability Studies Containing BH4 With or Without Antioxidant and whether Subjected to Gas Sparging or Not					
Study Group Number					
	1 Buffer Study	2 Buffer + Ascorbic Acid Study	3 Buffer + L- Cysteine in Study	5 Buffer + Argon Sparging Study	6 Buffer + Oxygen Sparging Study
1	1 mg/mL BH4 in pH 1.2 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 1.2 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 1.2 Buffer	1 mg/mL BH4 in pH 1.2 Buffer and Argon-Sparged and Argon blanket-Sealed	1 mg/mL BH4 in pH 1.2 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
2	1 mg/mL BH4 in pH 2.1 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 2.1 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 2.1 Buffer	1 mg/mL BH4 in pH 2.1 Buffer and Argon-Sparged and Argon blanket-Sealed	1 mg/mL BH4 in pH 2.1 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
3	1 mg/mL BH4 in pH 3 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 3 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 3 Buffer	1 mg/mL BH4 in pH 3 Buffer and Argon- Sparged and Argon blanket- Sealed	1 mg/mL BH4 in pH 3 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
4	1 mg/mL BH4 in pH 4 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 4 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 4 Buffer	1 mg/mL BH4 in pH 4 Buffer and Argon- Sparged and Argon blanket- Sealed	1 mg/mL BH4 in pH 4 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
5	1 mg/mL BH4 in pH 5 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 5 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 5 Buffer	1 mg/mL BH4 in pH 5 Buffer and Argon- Sparged and Argon blanket- Sealed	1 mg/mL BH4 in pH 5 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
6	1 mg/mL BH4 in pH 6 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 6 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 6 Buffer	1 mg/mL BH4 in pH 6 Buffer and Argon- Sparged and Argon blanket- Sealed	1 mg/mL BH4 in pH 6 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed
7	1 mg/mL BH4 in pH 7 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 7 Buffer	1 mg/mL BH4 and 1 mg/mL L-Cysteine in pH 7 Buffer	1 mg/mL BH4 in pH 7 Buffer and Argon- Sparged and Argon blanket- Sealed	1 mg/mL BH4 in pH 7 Buffer and Oxygen- Sparged and O <sub>2</sub> blanket-Sealed

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TABLE 17

Composition of Buffer Solutions for the pH 4 Stability Study of BH4	
pH 4	pH 4
Buffer + Ascorbic Acid + L-Cysteine Study	Buffer + Ascorbic Acid + L-Cysteine + Argon
1 mg/mL BH4 and 1 mg/mL Ascorbic Acid and 1 mg/mL L-Cysteine in pH 4 Buffer	Sparge Study 1 mg/mL BH4 + 1 mg/mL Ascorbic Acid + 1 mg/mL L-Cysteine in pH 4 Buffer and Argon-Sparged and Argon blanket-Sealed

TABLE 18

Composition of Buffer Solutions for the pH 7 Stability Study of BH4	
pH 7	pH 7
Buffer + Ascorbic Acid + L-Cysteine Study	Buffer + Ascorbic Acid + L-Cysteine + Argon
1 mg/mL BH4 and 1 mg/mL Ascorbic Acid and 1 mg/mL L-Cysteine in pH 7 Buffer	Sparge Study 1 mg/mL BH4 + 1 mg/mL Ascorbic Acid + 1 mg/mL L-Cysteine in pH 7 Buffer and Argon-Sparged and Argon blanket-Sealed

Proposed sampling times for studies to be conducted in various buffer solutions were estimated by comparing the half-life of a single study at pH 3.1 with data obtained by Davis, et al. (1988; *Eur. J. Biochem.* 173, 345-351, (1988)), in pH 6.8 Tris and phosphate buffers. The stability study of a pH 3.1 solution yielded an estimated  $t_{1/2}$  of 17769 min (12.3 days) and the work of Davis et al yielded a  $t_{1/2}$  of 10 min in phosphate pH 6.8 buffer and 14 min in pH 6.8 Tris buffer. These two studies suggest an order of magnitude reduction in half-life (i.e. an order of magnitude increase in reactivity) of BH4 for every one-fold increase in pH (see Table 19). Based on this approximation, pH 1.2 to pH 3 solutions were sampled weekly initially and sampling time corrections were made if necessary after the first 2 data points were collected. The estimated sampling times at 25° C. are provided in Table 19.

TABLE 19

Suggested Sampling Times at Various pH Based on Measured Half-life of BH4 and Theoretical Half-Lives Derived from Them			
pH	Measured $t_{1/2}$ (Min)	Estimated $t_{1/2}$ Based on $t_{1/2}$ Obtained at pH 3 (Min) <sup>a</sup>	Initially Suggested <sup>c</sup> Sampling Time
1.0	—	776900.0 (1234 days)	Every 7 days
2.0	—	177690.0 (123.4 days)	Every 7 days
3.0	17769.0 (12.34 days)	17769.0 (12.34 days)	Every 96 hours
4.0	—	1776.9 (1.23 days)	Every 12 hours
5.0	—	177.7 (0.12 days)	Every ½ Hour
6.0	—	17.7 (0.01 days)	Every 5 minutes <sup>d</sup>
6.8 <sup>b</sup>	10 (Phosphate) 14 (Tris)	—	—
7.0	—	1.8	Every ½ minutes <sup>d</sup>

<sup>a</sup>Estimated  $t_{1/2}$  is based on changing by an order of magnitude, the half-life obtained at pH 3.0 for every one-fold change in pH. pH < 3 are increased upwards while pH > 3 are decreased downwards by an order of magnitude in a stepwise fashion to roughly match the pH 6.8 data obtained by Davis et al.

<sup>b</sup>Data obtained from Davis, et al. 1988; *Eur. J. Biochem.*, 173, 345-351, (1988)

<sup>c</sup>Sampling can be modified

<sup>d</sup>Reaction solutions are sampled and quenched as fast as possible and require a stopwatch and 2 people, one sampling/quenching and the other accurately recording the time in a notebook in minutes and/or seconds

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Studies were conducted in pH 1-7 buffer solutions and at 5° C., 25° C., 30° C. and 37° C. Although these studies were conducted in non-hermetically sealed containers, anti-oxidants alone (ascorbic acid or L-cysteine) or combined together (ascorbic acid+L-cysteine) reduced the rate of loss or degradation of BH4 (see FIG. 36 and FIG. 37). Sparging a solution containing both ascorbic acid and L-cysteine substantially enhanced the stability of BH4.

The rate of degradation of BH4 is concentration-dependent (see FIG. 38). Therefore high dose, highly concentrated formulations of BH4 were shown to require lower concentration of stabilizers for synergistic stabilization of the formulations.

This results demonstrate that formulation of long shelf-life, stable, liquid formulations can be produced according to the methods and compositions described herein, including sterile injectable liquids, oral liquids, and lyophilized and sterile powders for constitution formulations.

## Example 7

## Liquid and Lyophilized Formulations of Tetrahydrobiopterin for Oral and Parenteral Use

## Example Compositions of Formulations

TABLE 20

Specific formulation buffered at pH 4 having ascorbic acid as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	10.00	1.00	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 21

Formulation buffered at pH 4.0 containing a combination of two stabilizers: ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	2.50	0.25	Antioxidant
Sodium Metabisulfite	2.50	0.25	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 22

Formulation buffered at pH 4.0 containing a combination of three stabilizers: L-cysteine, ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	2.00	0.20	Antioxidant
Sodium Metabisulfite	2.00	0.20	Antioxidant
L-Cysteine	4.00	0.40	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

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TABLE 23

Formulation buffered at pH 7.0 containing only ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	50.00	5.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.10	Buffering agent
Sodium Dibasic Phosphate	17.76	0.18	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 24

Formulation buffered at pH 7.0 containing ascorbic acid sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.26	Buffering agent
Sodium Dibasic Phosphate	17.76	0.44	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 25

Formulation buffered at pH 7.0 containing ascorbic, sodium metabisulfite and L-Cysteine as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
L-Cysteine	10.00	1.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.26	Buffering agent
Sodium Dibasic Phosphate	17.76	0.44	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

## High Dose Liquid Formulations

TABLE 26

Formulation buffered at pH 6.0 containing ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	0.10	Active substance
Ascorbic Acid	7.50	0.75	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

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TABLE 27

Formulation buffered at pH 6.0 containing a combination of two stabilizers: ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	5.00	Active substance
Ascorbic Acid	2.50	0.25	Antioxidant
Sodium Metabisulfite	2.50	0.25	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 28

Formulation buffered at pH 6.0 containing a combination of three stabilizers: L-cysteine, ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	0.10	Active substance
Ascorbic Acid	2.00	0.20	Antioxidant
Sodium Metabisulfite	2.00	0.20	Antioxidant
L-Cysteine	1.00	0.10	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 29

Oral formulation buffered at pH 3.0 citrate buffer and containing ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sucrose	200.00	20.00	Sweetener
Orange Flavor	1.00	0.10	Flavoring agent
Citric Acid	8.98	0.90	Buffering agent
Sodium Citrate, Dihydrate	2.13	0.21	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 30

Oral formulation buffered at pH 3.5 tartrate buffer and containing ascorbic acid and sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	5.00	0.50	Antioxidant
Sucrose	200.00	20.00	Sweetener
Grape Flavor	1.00	0.10	Flavoring agent
Tartaric Acid	1.34	0.13	Buffering agent
Sodium Tartrate Dibasic Dihydrate	8.39	0.84	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

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TABLE 31

Oral formulation buffered at pH 3.5 in malic acid based buffer and containing ascorbic acid and sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
Sucrose	200.00	20.00	Sweetener
Apple Flavor	1.00	0.10	Flavoring agent
Malic Acid	3.07	0.31	Buffering agent
Sodium Malate	4.91	0.49	Buffering agent
Dibasic			
Water for Injection qs	1.00 mL	1.00 mL	Diluent

The foregoing formulated or compounded solutions are optionally sparged with an inert gas (e.g., argon or nitrogen) or carbon dioxide in the compounding tank and primary containers preferably are sealed in a blanket of inert gas or carbon dioxide to remove oxygen from the container headspace. The formulations can be scaled up to any volume by multiplying the component amounts by an appropriate scale up factor.

## Example 8

LC/MS/MS Determination of Tetrahydrobiopterin (BH4) in Human Plasma by Measuring L-Biopterin Concentration upon Oxidation under Basic Conditions

Tetrahydrobiopterin (BH4) is a small molecule therapeutic for the treatment of patients with phenylketonuria (PKU). It is important to have an accurate and specific method to measure BH4 concentrations in human plasma. However, it is a challenge to quantify BH4 in human plasma because of its low endogenous concentration and instability. Under basic conditions, BH4 is oxidized into dihydrobiopterin (BH2) and ultimately L-Biopterin. Furthermore, the oxidation conversion ratio of BH4 to L-Biopterin is nearly constant up to 23 weeks. Therefore, by measuring L-Biopterin concentration upon oxidation under basic condition, and applying a molar conversion ratio, we can reliably determine the BH4 concentrations in human plasma.

Published methods are based on the classical method developed by Fukushima and Nixon (Anal. Biochem., 102, 176-188 (1980)) using HPLC with fluorescence detection. In the LC/MS/MS method, the human plasma sample was stabilized with antioxidant, spiked with an internal standard (IS) solution and basified with sodium hydroxide solution, then oxidized with iodine solution. Upon incubation in dark at room temperature, ascorbic acid is added to reduce the excess iodine. Oxidized samples were extracted by protein precipitation. L-Biopterin in the reconstituted extracts was analyzed by using reversed-phase HPLC with Turbo Ion Spray® MS/MS detection. Negative ions for L-Biopterin were monitored in MRM mode. Drug-to-IS peak area ratios for the standards were used to create a linear calibration curve using  $1/x^2$  weighted least-squares regression analysis.

The oxidation conversion ratio of BH4 to L-Biopterin was evaluated at multiple time-points: 0, 1, 2, 4, 8, 12 and 23 weeks, and found consistent in all the tested time-points with a nominal molar conversion ratio of 47.3% determined from the first three consecutive time-points. The difference between the conversion ratio at other time-points and the nominal value ranges from -2.3 to 6.3%. The LC/MS/MS

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method was validated to quantify L-Biopterin in K<sub>2</sub> EDTA human plasma in the linear calibration range of 5 to 1000 ng/mL (equivalent to 11 to 2114 ng/mL for BH4). The assay precision and accuracy was evaluated with quality control samples (QCs) and the results showed intraday precision between 4.7 to 14.5% CV; intraday accuracy between -7.1 to 7.4% nominal values; and interday precision and accuracy of 7.4 to 16.4% CV and -8.3 to 3.7% nominal values, respectively. The mean extraction recovery for L-Biopterin was 65.3%. In K<sub>2</sub> EDTA human plasma, L-Biopterin was found to be stable at room temperature for at least 4 hours and after 4 freeze thaw cycles, and at -70° C. for at least 275 days.

## Example 9

Determination of BH4/BH2/B Using HPLC with Electrochemical and Fluorescence Detection

A study was performed to develop a method of determining tetrahydrobiopterin (BH4), dihydrobiopterin (BH2) and biopterin (B) concentrations in human plasma using reverse phase high performance liquid chromatography (HPLC) with fluorescence detection (FD) and electrochemical detection (ECD). The method is based on Cai, et al. (*Cardiovascular Research* 55: 838-849, 2002).

Stock solutions of BH4 (in 20 mM HCl), BH2 and B (in DMSO) were made to a final concentration of 10 mM and stored at -80° C. Calibration standard working solutions were prepared from stock solution at 100, 10, 7.5, 5, 2.5, and 1 nM in K2 EDTA human plasma modified by 0.1% (w/v) 1,4-Dithioerythritol (DTE). Quality control working solutions of BH4, BH2 and B were prepared at 5, 8, 25 and 50 nM in K2 EDTA human plasma modified by 0.1% (w/v) DTE and stored at -80° C.

For sample processing, plasma was diluted 1:10 in resuspension buffer. To 180 µl of the diluted plasma, 20 µl of the 10x precipitation buffer was added. This process of plasma dilution and precipitation was applied to all plasma standards, plasma samples and plasma QCs. After the addition of the 10x precipitation buffer, the sample was centrifuged at maximum speed at 4° C. for 5 min to remove non-specific plasma debris. 150 mL supernatant was then be transferred to a sample vial and then placed on an autosampler for a 100 mL injection.

The mobile phase (2 L) was prepared with 13.6 g sodium acetate (50 mM), 2.1 g citric acid (5 mM) 36 mg EDTA (48 mM), 49.4 mg DTE (160 mM), and 2% methanol by volume in water. The pH was adjusted to 5.22. Resuspension buffer (20 mL) was made with 20 mL of PBS pH 7.4 (50 mM), 20 µL of 1 M DTE (1 mM), and 100 mL of 100 µM EDTA. The 10x precipitation buffer (25 mL) was made fresh with 2.88 mL phosphoric acid (1M), 9.39 g trichloroacetic acid (2 M) and 20 mL 1M DTE (1 mM).

Tetrahydrobiopterin (BH4), dihydrobiopterin (BH2), and Biopterin (B) were separated using reverse phase HPLC separation. BH4 was measured using electrochemical detection in which BH4 is oxidized by electrode 1 to quinonoid dihydrobiopterin (qBH2) and then reduced back to BH4 at electrode 2. The detector then uses the current generated by this reduction reaction to determine the concentration of BH4. BH2 and B can be measured in the same injection using fluorescence detection. Post column oxidation of BH2 using a conditioning guard cell at the optimum potential, oxidizes BH2 to Biopterin.

HPLC separation was carried out on an ACE C-18 (250 mm×4.6 mm) column, 5 µM, at a flow rate of 1.3 mL/min with a run time of 13 minutes. Electrochemical detection settings



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were E1: +100 mV (background current +500 nA to +600 nA) and E2: -300 mV (background current -50 nA to -60 nA). Post-column oxidation was set at 900 mV. Fluorescence detection settings were excitation wavelength: 350 nm and emission wavelength: 450 nm.

Linearity and range of the method were assessed based on the precision and accuracy of the standards in plasma and buffer. The standard curve concentration was established using at least 4-6 non-zero concentrations for each analyte. The concentration of the standards was 1, 2.5, 5, 7.5, 10, and 100 nM. The results showed a linear fit from 1 to 100 nM for BH4, BH2, and B with R2 of >0.99.

Accuracy was determined by replicate analysis of quality control samples containing known amounts (2, 8, 25, and 50 nM) of the analyte and expressed as a percent accuracy. Precision is also calculated based on the data from the quality controls. Intra-assay precision and inter-assay precision were evaluated based on the CV %. On three separate experimental runs concentrations of each analyte were prepared in plasma and analyzed. In addition 10 nM of BH4, BH2, and B was "spiked" into human plasma samples to determine the accuracy and recovery. The measurements of BH4, BH2, and B at 8, 25, and 50 nM proved accurate within 112%-89% and demonstrated precision (CV %) of 2.5%-20%. Spike recovery experiments using 10 nM BH4, BH2, and B in clinical samples of human plasma demonstrated recoveries between 70%-130%. The results demonstrate that the method is accurate and precise for samples with concentrations greater than 2 nM.

To check for the presence of endogenous interference in six different lots of plasma, 10 nM BH4, BH2, and B were spiked into six different lots of plasma and the determine accuracy and precision were determined for each plasma sample. Selectivity experiments show that the six individuals had endogenous baseline BH4 levels of between below quantifiable limit to 2.48 nM. Similarly, BH2 and B concentrations ranged from 0.02 to 10 nM. The recovery of the 10 nM spiked analytes ranged from 69%-87%. The variability (CV %) across the individual plasma samples and analytes when spiked at 10 nM ranged from 23%-37%. The variability of the endogenous levels of BH4, BH2 and B ranged from 0-9.96 nM. Together, the results indicate a trend suggesting matrix interference or loss during extraction, but do not indicate strong selectivity between individuals.

To measure matrix effect standard curves prepared in plasma or buffer were compared for accuracy (recovery), linearity and correlation. Comparison of the standards prepared in plasma versus standards prepared in buffer demonstrates a modest matrix effect and generally good correlation. All three analytes had excellent linear fits for plasma and buffer. BH4 and B did not demonstrate significant matrix effects across the concentration range. However, BH2 had less recovery at the highest standard concentration (100 nM). The quality control samples prepared in buffer and plasma demonstrated good accuracy. Overall, matrix effects seem minimal, with a trend toward less recovery in buffer as compared to plasma. Because BH4 and BH2 are readily oxidized, collected plasma and sample buffers should contain anti-oxidants and have low pH when possible.

To test the ability to accurately dilute a plasma and buffer sample spiked with 250 nM of BH4, BH2 and B, plasma was diluted using blank plasma in a 3-fold dilution series. The diluted samples were analyzed and compared to the nominal value after the dilution factor was applied. The dilution of high concentrations of BH4, BH2, and B can be accurately made. For BH4 the observed concentrations following dilution were between 83%-104% accurate for concentrations

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between 83.33 nM and 3.07 nM. BH2 was 74%-80% accurate across the quantitative range (83 nM-3 nM). B was 119%-113% accurate across the quantitative range (83 nM-3 nM). Therefore, a sample that is above the quantitative limit can be diluted accurately.

Four concentrations of analytes (2, 8, 25 and 50 nM) were prepared in plasma and frozen for a minimum of 24 hours for one cycle and a minimum of 12 hours for other cycles for a minimum of three cycles. Samples were thawed unassisted at room temperature in between frozen periods. The accuracy and variability after each and all free-thaw cycles was assessed to establish the maximal number of cycles a sample could undergo. The BH4-, BH2-, and B-containing samples can undergo up to 3 freeze-thaw cycles without significant change in accuracy or precision of the measurement. Plasma samples with 8 nM-50 nM BH4 are 121%-91% accurate and CV % less than 10%. Similarly BH2 measurements were 77%-88% accurate across the quantitative range of the assay. B measurements were 98%-99% accurate across the quantitative range with precision (CV %) of 5%-8%. The 2 nM sample of BH4, BH2, and B did not prove accurate or precise following repeated freeze-thaw. Therefore, standards, quality controls and study samples may be frozen and thawed up to 3 times.

Because the analytes are sensitive to oxidation we examined long-term frozen stability to mimic expected storage conditions. Four concentration levels (2, 8, 50, and 100 nM) of BH4, BH2, and B were prepared in plasma and stored at -70° C. for 8 weeks. Stability samples were assayed fresh and at weeks 3, 5, 6, and 8. BH4 and B had good long term frozen stability. BH2 demonstrated reduced sample concentration after prolonged storage. Over the 8 weeks of storage, plasma samples with BH4 were 93%-94% accurate and had CV % between 31%-0.21%, with the most variation seen at the 2 nM concentration. BH2 measurements were 63%-85% accurate across the concentrations tested with reduced accuracy at the 2 nM and 100 nM concentrations. The precision (CV %) ranged from 37% to 18% for these samples. B measurements were 88%-101% accurate across the concentrations tested with precision (CV %) of 23%-0.14%, with the highest variability at the 2 nM concentration. Together, this data supports the recommendation to store samples for up to 8 weeks without appreciable loss of analyte concentration. BH2 seems to be the most susceptible to degradation (oxidation).

To measure the stability of BH4, BH2, and B in the autosampler, 8 nM of each analyte in reconstitution solvent stayed on the autosampler for 0.25, 4, and 11 hours. The accuracy and precision of the measurements were compared. The observed BH4 measurement was accurate within 5% of theoretical at each time point with accuracy and precision across all three measurements of 102% and 0.054% respectively. The measurement of BH2 had decreasing accuracy and increasing variability after 4 hours. After 11 hours on the autosampler about 50% of the BH2 was measured. This indicates poor autosampler stability in run buffer. The measurement of B remained accurate within 125% of theoretical after 11 hours. Therefore, run times of no more than 4 hours are recommended.

To determine injection carry-over, an extracted baseline plasma sample was inserted after the highest standard concentration 100 nM. This was done to mimic the possibility of overestimating the concentration of analyte in a low concentration sample due to carry-over. The injection carryover of BH4, BH2, and B is minimal and does not account for more than 1% of the peak area of the 100 nM upper limit of quantitation. The injection carryover accounts for approximately 5%-20% of the lower limit of quantitation, based on the

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average peak area obtained from the low quality control (2 nM). Therefore, preferably the samples should be ordered from lowest to high (i.e., pre-dose first, followed by post-dose samples) and additional washes to clean the column periodically during a run preferably will be made to minimize potential carryover.

A qualified method which was robust, specific, accurate and precise was developed. This method is appropriate to quantify the levels of BH4, BH2 and B in plasma for pharmacokinetic and drug studies.

All patents, publications and references cited herein are hereby fully incorporated by reference. In case of conflict between the present disclosure and incorporated patents, publications and references, the present disclosure will control.

What is claimed is:

1. A method of treating hyperphenylalaninemia (HPA) comprising orally administering to a patient diagnosed with HPA a therapeutically effective amount of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, at a daily dose of 5 to 20 mg/kg and for a duration of at least 3 weeks or longer, wherein said administering comprises (i) dissolving a tablet or capsule of said BH4 or pharmaceutically acceptable salt thereof in liquid, and separately (ii) taking the dissolved BH4 or pharmaceutically acceptable salt thereof orally with food, at the same time as the food or within approximately 30 minutes after the food.

2. The method of claim 1 further comprising informing said human that absorption of said BH4 or pharmaceutically acceptable salt thereof is increased when it is ingested with food.

3. The method of claim 1, further comprising informing the human that both the mean maximum plasma concentration ( $C_{max}$ ) and area under the plasma concentration-time curve (AUC) of BH4 are increased when the BH4 or pharmaceutically acceptable salt thereof is administered with a high-fat, high-calorie meal, compared to when the BH4 or pharmaceutically acceptable salt thereof is administered under fasted conditions.

4. The method of claim 3, wherein the increases in  $C_{max}$  and AUC are increases of at least 30%.

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5. The method of claim 1, wherein said BH4 is a crystalline polymorph, as a hydrochloride salt, that exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 5.63 (m), 4.76 (m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w).

6. The method of claim 1, wherein the human has been diagnosed with HPA due to BH4-responsive phenylketonuria (PKU).

7. The method of claim 1, wherein the human has been diagnosed with HPA due to BH4 deficiency.

8. The method of claim 1, wherein the frequency of administering the BH4 or pharmaceutically acceptable salt thereof consists of once daily administration.

9. A method of increasing absorption of tetrahydrobiopterin (BH4), comprising orally administering to a human in need thereof a therapeutically effective amount of BH4 or a pharmaceutically acceptable salt thereof dissolved in a liquid, at the same time as or within approximately 30 minutes after a high fat, high calorie meal, to increase absorption of BH4.

10. The method of claim 9, wherein the increase in absorption of said BH4 is characterized by increases in mean maximum plasma concentration ( $C_{max}$ ) and area under the plasma concentration-time curve (AUC) of at least about 30%.

11. A method of treating hyperphenylalaninemia (HPA) comprising orally administering to a patient diagnosed with HPA a daily dose of 5 to 20 mg/kg of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, wherein said administering comprises (i) dissolving a tablet or capsule of said BH4 or pharmaceutically acceptable salt thereof in liquid, and separately (ii) taking the dissolved BH4 or pharmaceutically acceptable salt thereof orally, at the same time as or within approximately 30 minutes after a high fat, high calorie meal.

\* \* \* \* \*

# EXHIBIT D

US008003126B2

(12) **United States Patent**  
**Jungles et al.**(10) **Patent No.:** **US 8,003,126 B2**  
(45) **Date of Patent:** **\*Aug. 23, 2011**(54) **STABLE TABLET FORMULATION**(75) Inventors: **Steven Jungles**, Novato, CA (US);  
**Mark Henderson**, Larkspur, CA (US);  
**Victoria Sluzky**, Corte Madera, CA  
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CA (US)(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 1030 days.This patent is subject to a terminal dis-  
claimer.(21) Appl. No.: **10/563,418**(22) PCT Filed: **Nov. 16, 2005**(86) PCT No.: **PCT/US2005/041252**

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(2), (4) Date: **Jul. 24, 2007**(87) PCT Pub. No.: **WO2006/055511**PCT Pub. Date: **May 26, 2006**(65) **Prior Publication Data**

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17, 2004.(51) **Int. Cl.**  
**A61K 9/20** (2006.01)(52) **U.S. Cl.** ..... **424/464**(58) **Field of Classification Search** ..... None  
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*Primary Examiner* — Michael G Hartley*Assistant Examiner* — James Rogers(74) *Attorney, Agent, or Firm* — Jones Day(57) **ABSTRACT**

The present invention is directed to a stable solid formulations of tetrahydrobiopterin, processes for producing them, and treatment methods using such formulations.

**22 Claims, 1 Drawing Sheet**

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U.S. Patent

Aug. 23, 2011

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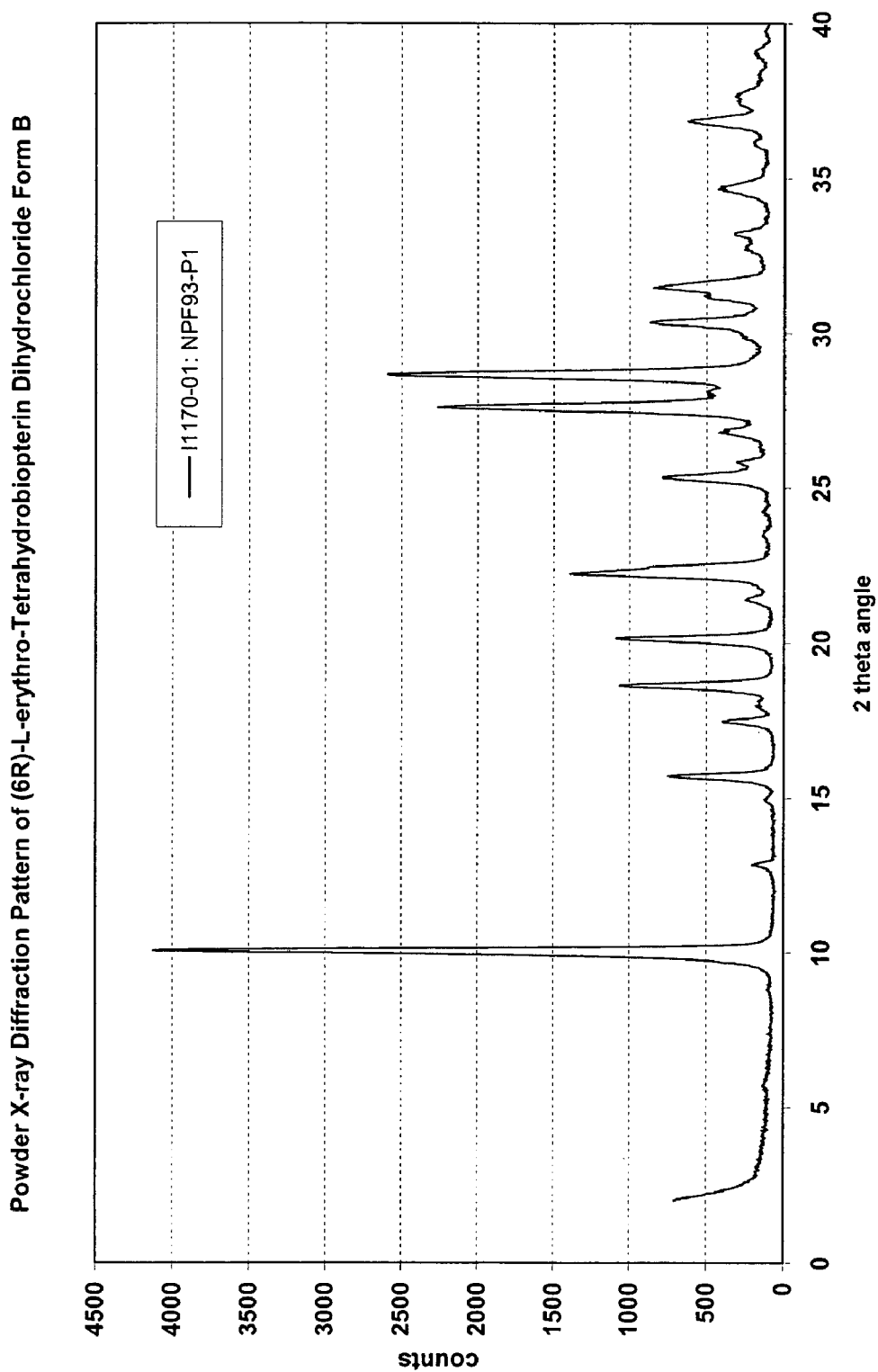


FIGURE 1

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**STABLE TABLET FORMULATION****CROSS-REFERENCE TO RELATED APPLICATION**

This is the U.S. national phase application of International Application No. PCT/US2005/041252, filed Nov. 16, 2005, which claims the benefit of U.S. Provisional Application No. 60/629,189, filed Nov. 17, 2004.

**BACKGROUND****1. Field**

The present invention is generally directed to stable tablet formulations of tetrahydrobiopterin or precursors, derivatives or analogs thereof for the treatment of humans.

**2. Background of the Related Technology**

Tetrahydrobiopterin (sometimes referred to as BH4) is a biogenic amine of the naturally-occurring pterin family that is a cofactor for a number of different enzymes, including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. It is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (*Disorders of tetrahydrobiopterin and related biogenic amines*. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1275-1776).

**Names of Parties to a Joint Research Agreement**

BioMarin Pharmaceutical Inc. and Merck Eprova AG

A deficiency in PAH activity, due to absent or mutated PAH enzyme or a deficiency in its cofactor BH4, manifests as an excess of the amino acid phenylalanine (Phe) known as hyperphenylalaninemia (HPA) in its very mildest forms or phenylketonuria (PKU) in its moderate or severe forms. PAH deficiency also causes a deficiency in the amino acid tyrosine, which is a precursor for synthesis of neurotransmitters. A deficiency in tyrosine hydroxylase or tryptophan hydroxylase activity can manifest as a deficiency in neurotransmitter production.

Despite the elucidation of the role of BH4 deficiency in phenylketonuria, treatment with BH4 has not been suggested because such treatment is very expensive, as high as \$30,000 per year for an adolescent or adult, as compared with \$6,000 for phenylalanine-restricted dietary therapy (Hanley, *N. Engl. J. Med* 348(17):1723, 2003). Another significant problem with BH4 is that this compound is unstable and readily undergoes aerobic oxidation at room temperature (Davis et al., *Eur. J. Biochem.*, Vol 173, 345-351, 1988; U.S. Pat. No. 4,701, 455) and has a shelf-life of less 8 hours at room temperature (Bernegger and Blau, *Mol. Genet. Metabol.* 77:304-313, 2002).

Other tetrahydrobiopterin products available on the market need to be specially packaged or kept frozen. For example the labeling on the tablets sold by Schirck's Laboratory specify that the tablets should be kept frozen and state that the product has a shelf life at room temperature of only 2 months. BIOPTEN (tetrahydrobiopterin granules) requires expensive, hermetically-sealed foil packaging to maintain room temperature stability. The instability of such BH4 compositions is

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commercially undesirable and significant degradation due to improper storage could hinder therapy of patients.

Drug substance polymorphic forms can exhibit different physical and mechanical properties, including hygroscopicity, particle shape, density, flowability, and compactibility, which in turn may affect processing of the drug substance and/or manufacturing of the drug product. The effect of polymorphism on pharmaceutical processing also depends on the formulation and the manufacturing process. Polymorphic forms of the drug substance can undergo phase conversion when exposed to a range of manufacturing processes, such as drying, milling, micronization, wet granulation, spray-drying, and compaction. Exposure to environmental conditions such as humidity and temperature can also induce polymorph conversion. The extent of conversion generally depends on the relative stability of the polymorphs, kinetic barriers for phase conversion, and applied stress. See FDA Center for Drug Evaluation and Research (CDER) Draft Guidance for Industry ANDAs: Pharmaceutical Solid Polymorphism Chemistry, Manufacturing, and Controls Information, December 2004.

Thus, there remains a need for a stable solid formulation of tetrahydrobiopterin and processes for manufacturing such stable formulations. The present invention is directed to addressing such a need.

**SUMMARY OF THE INVENTION**

The present invention relates to stable solid formulations of tetrahydrobiopterin, particularly stable tablets, processes for producing such formulations, and treatment methods using such formulations.

The invention provides a stable solid formulation of tetrahydrobiopterin, or a precursor or derivative or analog thereof, that maintains its stability for an extended period of time. Compositions of the invention may comprise a stable, crystalline form of BH4 that is stable at room temperature for more than 8 hours and a pharmaceutically acceptable carrier, diluent or excipient. Exemplary stable tablets of the invention have been prepared using a dry tableting process and have been shown to have a shelf-life of at least 6 to 9 months at room temperature.

Another aspect of the invention provides a dry formulation process for preparing stable solid formulations, which includes the step of mixing tetrahydrobiopterin, or a precursor or derivative or analog thereof, with another pharmaceutical carrier, diluent or excipient, in the absence of added water.

In an exemplary embodiment, the active pharmaceutical ingredient and excipients are dry blended and compressed. The tablets are processed in humidity-controlled rooms where humidity is kept at about 65% ( $\pm 5\%$ ) or less. Once processed, the tablets are stored in triple plastic lined water resistant containers with desiccant pillows lined between the outer two layers of plastic bags. Thus, the invention includes a dry formulation method comprising the steps of mixing an initial amount of a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin, preferably polymorph B, and one or more pharmaceutically acceptable excipients, and forming a tablet from the mixture, wherein the steps do not include adding liquid water. Exemplary particle sizes include, e.g., from about 0.2  $\mu\text{m}$  to about 500  $\mu\text{m}$ , from about 1  $\mu\text{m}$  to about 250  $\mu\text{m}$ , or from about 2  $\mu\text{m}$  to about 200  $\mu\text{m}$ , or, e.g., smaller than about 500  $\mu\text{m}$ , smaller than about 600  $\mu\text{m}$ , smaller than about 700  $\mu\text{m}$ , or smaller than about 850  $\mu\text{m}$ .

In exemplary embodiments, the tablet is initially manufactured using a stable crystalline form of (6R)-5,6,7,8-tetrahy-



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drobiopterin described below as "polymorph B," and retains at least about 95% of the active pharmaceutical ingredient (API) at room temperature after 3 months, 6 months or 9 months, or preferably 12 months or longer, e.g. 15 months, 18 months, 21 months, 2 years, 2.5 years, 3 years or longer. Preferably the tablet retains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% of the API after storage at room temperature for such time periods. The tablet also preferably exhibits loss on drying of 2% or less, or 1.5% or less, or 1% or less, or 0.9% or less, or 0.8% or less, or 0.7% or less, or 0.6% or less, after such time periods. Exemplary tablets may be manufactured wherein the initial amount of tetrahydrobiopterin active pharmaceutical ingredient is about 25 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, 500 mg, 550 mg, 600 mg, 650 mg, 700 mg, 750 mg, 800 mg, 850 mg, 900 mg or higher doses. Preferred tablets also exhibit rapid disintegration upon administration, e.g., 3 minutes or less, to improve ease of administration.

Thus, the invention provides a stable tablet formulation comprising an initial amount of a crystalline polymorph, designated polymorph B, of (6R)-L-erythro-tetrahydrobiopterin and a pharmaceutically acceptable excipient, wherein after six months at room temperature and about 60% humidity the stable tablet formulation retains at least about 95% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin, and wherein said crystalline polymorph, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A) : 8.7 (vs), 5.63 (m), 4.76(m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w). Preferably the tablet retains at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% of the initial amount of (6R)-L-erythro-tetrahydrobiopterin.

The stable solid formulation preferably contains one or more of the following additional ingredients that improve stability or other characteristics of the formulation: binder, disintegrant, acidic antioxidant, or lubricant or combinations thereof. One exemplary preferred composition includes anhydrous dibasic calcium phosphate, crospovidone, ascorbic acid and stearyl fumarate, optionally with mannitol and riboflavin. The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or vitamins such as vitamin C and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan; and/or arginine. Compositions comprising tetrahydrobiopterin (or a precursor or derivative or analog) and a folate, and optionally further comprising arginine, are particularly contemplated.

The invention further contemplates other stable solid formulations for oral administration, e.g. capsules, pills or troches, with similar stability properties.

Yet another aspect of the invention provides treatment methods using such stable solid formulations. The invention contemplates that such formulations of the invention are useful for intervention in metabolic disorders, particularly those involving amino acid metabolism. More particularly, the stable formulations may be used for the treatment of subjects exhibiting elevated phenylalanine levels or decreased tyrosine levels, for example, subjects suffering from hyperphenylalanemia, mild phenylketonuria or classic severe phenylketonuria; and for the treatment of subjects suffering from

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conditions that would benefit from enhancement of nitric oxide synthase activity, including vascular diseases, ischemic or inflammatory diseases, diabetes, or insulin resistance. The total dose required for each treatment may be administered in multiple doses or in a single dose. The stable formulations may be administered daily or at some other interval, e.g., every alternative day or even weekly.

The stable formulations may be used alone or in conjunction with other therapies suitable for the disorder to be treated, including the underlying disease or the clinical symptoms. For example, for HPA, the stable formulations of the invention may be administered in combination with a protein-restricted diet, e.g. where the subject is limited to about 600 mg or less, or about 300 mg or less of protein daily, optionally with supplemental amino acids, such as tyrosine, valine, isoleucine and leucine. The stable formulations may also be administered in combination with folates, arginine, vitamins, or neurotransmitter precursors. As another example, for vascular diseases, diabetes, or insulin resistance, the stable formulations of the invention may be administered in conjunction with other therapeutic agents such as anti-hypertensive agents, anti-platelet agents, cholesterol-lowering agents, insulin or oral hypoglycemic agents.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a characteristic X-ray powder diffraction pattern for crystalline polymorph B of (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a stable formulation that maintains a stable crystalline polymorph of the active ingredient. An anhydrous polymorph of (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride that is stable at room temperature to atmospheric oxygen and normal humidity, described below as polymorph B, has been identified. However, when the percent relative humidity approaches 80%, polymorph B appears to take up much more water, loses its crystalline form, and becomes labile to oxidation.

By using a dry formulation process, the stable crystalline structure of this polymorph is maintained in the finished product. In contrast, other processes for preparing tetrahydrobiopterin compositions result in a less stable product compared to those of the present invention.

Stable tablet formulations of the invention have been made using polymorph B in a dry formulation process and have been shown to retain 99% or more of the initial (6R)-5,6,7,8-tetrahydrobiopterin for at least 6 or 9 months, both at normal room temperature and humidity, and under accelerated testing conditions. The observed stability under accelerated testing conditions, i.e. higher temperature and humidity, indicates that the tablet formulations would be stable for far longer than 6 or 9 months at normal room temperature and humidity.

As used herein, "shelf life" means the storage period during which an active pharmaceutical ingredient (API) in a

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pharmaceutical formulation has minimal degradation (e.g., not more than about 5% degradation) when the pharmaceutical formulation is stored under specified storage conditions, for example, room temperature at normal humidity.

The shelf-life of the stable formulations of the invention may be measured as follows. The formulation to be tested may be divided into one or more different batches and stored under typical storage conditions, for example, 4° C. (refrigerator), or 25° C. (room temperature). Degradation of the API in a pharmaceutical formulation can also be detected using accelerated testing under exaggerated storage conditions designed to increase the degradation rate of the drug substance. For example, a batch can be "stressed" (placed in chamber which maintains a temperature of 45° C. and 75% humidity). Samples of each batch of formulation are then analyzed at different time points (e.g., time zero, 2 weeks, 1 month, 3 months, 6 months, 9 months, 1 year, 1.5 years, 2 years, 2.5 years, 3 years or longer) for amount of API still present in the formulation. Analysis of the API in the formulation may be carried out by a variety of detection methods including high performance liquid chromatography, crystal or powder X-ray diffraction, infrared or Raman spectra studies, microscopy, differential scanning calorimetry, thermal gravimetric analysis, hot-stage microscopy, and solid state nuclear magnetic resonance. Maintenance of a particular polymorph form can be determined by carrying out, e.g., powder or crystal X-ray diffraction studies or any of the same techniques used to analyze the polymorph initially.

#### I. Synthesis of Tetrahydrobiopterin, Precursors, Derivatives and Analogs

A variety of methods are known in the art for synthesis of tetrahydrobiopterins, precursors, derivatives and analogs. U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408, Canadian application CA 2420374, European application nos. EP 079 574, EP 191 335 and Suntory Japanese patent publications JP 4-082888, JP 59-021685 and JP 9-157270, as well as Sugimoto and Matsuura, *Bull. Chem. Soc. Japan*, 48(12):3767-3768 (1975), Sugimoto and Matsuura, *Bull. Chem. Soc. Japan*, 52(1):181-183 (1979), Matsuura et al., *Chem. Lett. (Japan)*, 735-738 (1984), Matsuura et al., *Heterocycles*, Vol. 23, No. 12, 3115-3120, 1985 and Whiteley et al., *Anal Biochem.* 137(2):394-6 (1984) (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivatives thereof that may be used as compositions for the present invention.

Int'l Publication No. WO2005049614, U.S. Pat. No. 4,540,783, Japanese Patent No. 59-021685, Schircks et al., *Helv. Chim. Acta*, 60: 211 (1977), Sugimoto et al., *Bull. Chem. Soc. Jp*, 52(1):181 (1979), Sugimoto et al., *Bull. Chem. Soc. Jp*, 48(12):3767 (1975), Visontini et al., *Helv. Chim. Acta*, 52:1225 (1969), and Matsuura et al., *Chem. Lett.*, p 735 (1984), incorporated herein by reference in their entireties, describe methods of synthesizing BH4.

Nonlimiting examples of analogs for use in the compositions and methods described herein include pteridine, pterin, neopterin, biopterin, 7,8-Dihydrobiopterin, 6-methyltetrahydropterin, and other 6-substituted tetrahydropterin and other 6-substituted tetrahydropterins, sepiapterin, 6,7-Dimethyltetrahydropterin, 6-methyl biopterin and other 6-substituted biopterins, and other analogs that are described in the art. Nonlimiting examples of derivatives for use in the compositions and methods described herein include the derivatives described in U.S. Pat. Nos. 4,758,571; 4,774,244; 6,162,

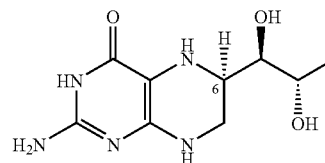
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806; 5,902,810; 2,955,110; 2,541,717; 2,603,643; and 4,371,514, the disclosures of which are hereby incorporated herein.

Any such methods or other methods known in the art may be used to produce BH4, or precursors, derivatives or analogs for use in the stable formulations and therapeutic methods of the present invention.

#### II. Crystal Polymorphs of 6R-tetrahydrobiopterin Hydrochloride Salt

It has been found that BH4, and in particular, the dihydrochloride salt of BH4, exhibits crystal polymorphism. The structure of BH4 is shown below:



The (6R) form of BH4 is the known biologically active form, however, BH4 is known to be unstable at ambient temperatures.

BH4 is difficult to handle and it was therefore produced and offered as its dihydrochloride salt (Schircks Laboratories, Jona, Switzerland) in ampoules sealed under nitrogen to prevent degradation of the substance due to its hygroscopic nature and sensitivity to oxidation. U.S. Pat. No. 4,649,197 discloses that separation of (6R)- and 6(S)-L-erythro-tetrahydrobiopterin dihydrochloride into its diastereomers is difficult due to the poor crystallinity of 6(R,S)-L-erythro-tetrahydrobiopterin dihydrochloride. The European patent number 0 079 574 describes the preparation of tetrahydrobiopterin, wherein a solid tetrahydrobiopterin dihydrochloride is obtained as an intermediate. S. Matsuura et al. describes in *Chemistry Letters* 1984, pages 735-738 and *Heterocycles*, Vol. 23, No. 12, 1985 pages 3115-3120 6(R)-tetrahydrobiopterin dihydrochloride as a crystalline solid in form of colorless needles, which are characterized by X-ray analysis disclosed in *J. Biochem.* 98, 1341-1348 (1985). An optical rotation of 6.81° was found the crystalline product, which is quite similar to the optical rotation of 6.51° reported for a crystalline solid in form of white crystals in example 6 of EP-A2-0 191 335.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride indicated that the compound may exist in different crystalline forms, including polymorphic forms and solvates. It has been found that one crystal polymorph of BH4 is more stable, and is stable to decomposition under ambient conditions.

#### Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B."

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties renders polymorph form B especially feasible for pharmaceutical applications, which are often prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.



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Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride. As used herein, the following abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity.

Form B can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive crystal form B, can be used for the preparation of the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms, in suitable polar and non aqueous solvents.

Other forms of BH4 can be converted into form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methyl-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallisation from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water:acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallization from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallisation and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

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In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application.

### III. Stable Pharmaceutical Formulations

Pharmaceutical formulations may initially include a stable crystalline form of tetrahydrobiopterin, or a precursor or derivative or analog thereof, with a pharmaceutically acceptable carrier. The stable formulation of the invention preferably contains one or more of the following additional ingredients that improve the stability or other characteristics of the formulation: binder, disintegration agent, acidic antioxidant, or lubricant or combinations thereof. Preferably a stable tablet formulation includes a binder and disintegration agent, optionally with an acidic antioxidant, and optionally further including a lubricant.

The initial amount of a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin used to prepare the formulation may be, for example, in the range of about 30 wt % to about 40 wt % of the formulation, or in the range of about 32 wt % to about 35 wt %, or at about 33 wt %.

Binders assist in maintaining the tablet formulation. In some cases, anhydrous binders are used to preserve the anhydrous state of polymorph B. In some cases, the binder may act as a drying agent. Exemplary binders include anhydrous dibasic calcium phosphate and its monohydrate.

Exemplary concentrations of the binder in a stable tablet formulation of the present invention are between about 1 wt % to about 5 wt %. Particularly contemplated concentrations are between about 1.5 and 3 wt %. Also contemplated are concentrations of binder of at least about 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %, or concentrations up to about 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, and 5.0 wt %. The weight ratio of binder to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:10 to about 1:20. Also contemplated are weight ratios of about 1:10.25, 1:10.5, 1:10.75, 1:11, 1:11.25, 1:11.5, 1:11.75, 1:12, 1:12.25, 1:12.5, 1:12.75, 1:13, 1:13.25, 1:13.5, 1:13.75, 1:14, 1:14.25, 1:14.5, 1:14.75, 1:15, 1:15.25, 1:15.5, 1:15.75, 1:16, 1:16.25, 1:16.5, 1:16.75, 1:17, 1:17.25, 1:17.5, 1:17.75, 1:18, 1:18.25, 1:18.5, 1:18.75, 1:19, 1:19.25, 1:19.5, and 1:19.75.

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Disintegration agents assist in rapid disintegration of solid formulations by absorbing water and expanding. Exemplary disintegration agents include polyvinylpyrrolidone (PVP, e.g. sold under the name POVIDONE), a cross-linked form of povidone (CPVP, e.g. sold under the name CROSPOLV-DONE), a cross-linked form of sodium carboxymethylcellulose (NaCMC, e.g. sold under the name AC-DI-SOL), other modified celluloses, and modified starch. Tablets formulated with CPVP exhibited much more rapid disintegration than tablets formulated with PVP.

Exemplary concentrations of the disintegration agent in a stable table formulation of the present invention are between about 1 wt % to about 20 wt %. Particularly contemplated concentrations are between about 3 wt % and about 10 wt %. Also contemplated are concentrations of disintegration of at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %, or concentrations up to about 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.2, 5.4, 5.6, 5.7, 5.8, 6.0, 6.25, 6.5, 6.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5, 9.75, and 10.0 wt %. The weight ratio of disintegration agent to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:5 to about 1:10. Also contemplated are weight ratios of about 1:5.25, 1:5.5, 1:5.75, 1:6.0, 1:6.25, 1:6.5, 1:6.75, 1:7.0, 1:7.25, 1:7.5, 1:7.75, 1:8.0, 1:8.25, 1:8.5, 1:8.75, 1:9.0, 1:9.25, 1:9.5, and 1:9.75.

Antioxidants may be included and help stabilize the tetrahydrobiopterin product, especially after dissolution. Low pH aqueous solutions of API are more stable than are solutions at high pH. Exemplary acidic antioxidants include ascorbic acid, fatty acid esters of ascorbic acid such as ascorbyl palmitate and ascorbyl stearate, and salts of ascorbic acid such as sodium, calcium, or potassium ascorbate. Non-acidic antioxidants may also be used in the stable tablet formulations. Nonlimiting examples of non-acidic antioxidants include beta-carotene, alpha-tocopherol. Acidic additives may be added to enhance stability of the tablet formulation, including citric acid or malic acid.

Exemplary concentrations of the antioxidant in a stable table formulation of the present invention are between about 1 wt % and about 3 wt %. Particularly contemplated concentrations are at least about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, and 2.0 wt %, or concentrations up to about 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, and 3.0 wt %. The weight ratio of antioxidant to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:5 to 1:30. Also contemplated are weight ratios of about 1:5.5, 1:6, 1:6.5, 1:7, 1:7.5, 1:8, 1:8.5, 1:9, 1:9.5, 1:10, 1:10.5, 1:11, 1:11.5, 1:12, 1:12.5, 1:13, 1:13.5, 1:14, 1:14.5, 1:15, 1:15.5, 1:16, 1:16.5, 1:17, 1:17.5, 1:18, 1:18.5, 1:19, 1:19.5, 1:20, 1:20.5, 1:21, 1:21.5, 1:22, 1:22.5, 1:23, 1:23.5, 1:24, 1:24.5, 1:25, 1:25.5, 1:26, 1:26.5, 1:27, 1:27.5, 1:28, 1:28.5, 1:29, and 1:29.5.

In Schirk's Laboratory tablets, ascorbic acid is present at a ratio of 1:1 to BH4. In contrast, the concentration of ascorbic acid in the stable formulations of the invention is far lower, e.g. a weight ratio (mg/mg) of 1:20 of ascorbic acid to BH4. Thus, the invention also contemplates formulations comprising ascorbic acid at a ratio to BH4 of less than 1:1, e.g. 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18 or 1:19, preferably a ratio of less than 1:10.

Lubricants improve stability, hardness and uniformity of solid formulations. Exemplary lubricants include stearyl fumarate and magnesium stearate.

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Exemplary concentrations of the lubricant in a stable tablet formulation of the present invention are between about 0.1 wt % and about 2 wt %. Particularly contemplated concentrations are between about 0.5 and 1 wt %. Also contemplated are concentrations of lubricant of at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 wt %, or concentrations up to about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, and 2.0 wt %. The weight ratio of lubricant to tetrahydrobiopterin in a stable tablet formulation of the present invention is, for example, in the range of about 1:25 to 1:65. Also contemplated are weight ratios of about 1:26, 1:27, 1:28, 1:29, 1:30, 1:31, 1:32, 1:33, 1:34, 1:35, 1:36, 1:37, 1:38, 1:39, 1:40, 1:41, 1:42, 1:43, 1:44, 1:45, 1:46, 1:47, 1:48, 1:49, 1:50, 1:51, 1:52, 1:53, 1:54, 1:55, 1:56, 1:57, 1:58, 1:59, 1:60, 1:61, 1:62, 1:63, 1:64, and 1:65.

The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or arginine; and/or vitamins, such as vitamin C and/or vitamin B2 (riboflavin) and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan.

Exemplary folates, including folate precursors, folic acids, or folate derivatives, are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folylpolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folylpolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and pharmaceutically acceptable salts thereof. Exemplary salts include sodium, potassium, calcium or ammonium salts.

Exemplary relative weight ratios of BH4 to folates to arginine may be from about 1:10:10 to about 10:1:1.

Optionally the stable formulations of the invention can also comprise other excipients such as mannitol, hydroxyl propyl cellulose, microcrystalline cellulose, or other non-reducing sugars such as sucrose, trehalose, melezitose, planteose, and raffinose. Reducing sugars may react with BH4.

Pharmaceutically acceptable ingredients for manufacturing solid formulations for oral administration may be incorporated, for example, by admixing the components and optionally finely dividing them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches. Coatings may be applied after compression to form pills.

Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be for example binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. Nonlimiting examples of binders useful in a composition described herein include gum tragacanth, aca-

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cia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (polyoxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Nonlimiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate. Nonlimiting examples of lubricants use in a composition described herein include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Surfactants for use in a composition described herein can be anionic, anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The polymorph described herein may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. Slow release formulations may also be prepared from the polymorph described herein in order to achieve a controlled release of the active agent in contact with the body fluids in the gastrointestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release

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formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used. Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N' dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4 aminosalicic acid, 2 phenoxybenzoic acid, 2 acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzenesulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2 sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

The stable formulations of the invention may be provided, e.g. as tablets or pills or capsules in HDPE bottles provided with a desiccant capsule or pouch; or in foil-on-foil blister packaging, or in blister packaging comprising see-through polymer film, if commercially desirable.

The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the



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therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey ducks and geese.

#### IV. Treatment Methods Using Stable Formulations

The stable formulations of the invention may be used for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine or tryptophan levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal. Conditions associated with decreased tyrosine or tryptophan levels include neurotransmitter deficiency, neurological and psychiatric disorders such as Parkinson's, dystonia, spinocerebellar degeneration, pain, fatigue, depression, other affective disorders and schizophrenia.

The stable formulations may also be used for treating patients suffering from BH4 deficiency, e.g., due to a defect in the pathway for its synthesis, including but not limited to dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, or dihydropteridine reductase (DHPR) deficiency.

Suitable subjects for treatment with the stable formulations of the invention include subjects with an elevated plasma Phe concentration in the absence of the therapeutic, e.g. greater than 1800  $\mu\text{M/L}$ , or greater than 1600  $\mu\text{M}$ , greater than 1400  $\mu\text{M}$ , greater than 1200  $\mu\text{M}$ , greater than 1000  $\mu\text{M}$ , greater than 800  $\mu\text{M}$ , or greater than 600  $\mu\text{M}$ , greater than 420  $\mu\text{M}$ , greater than 300  $\mu\text{M}$ , greater than 200  $\mu\text{M}$ , or greater than 180  $\mu\text{M}$ . Mild PKU is generally classified as plasma Phe concentrations of up to 600  $\mu\text{M/L}$ , moderate PKU as plasma Phe concentrations of between 600  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$  and classic or severe PKU as plasma Phe concentrations that are greater than 1200  $\mu\text{M/L}$ . Preferably treatment with the stable formulations alone or with protein-restricted diet decreases the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ , or less than 500  $\mu\text{M}$ , or 360  $\mu\text{M} \pm 15 \mu\text{M}$  or less, or less than 200  $\mu\text{M}$ , or less than 100  $\mu\text{M}$ . Other suitable subjects include subjects diagnosed as having a reduced phenylalanine hydroxylase (PAH) activity. Reduced PAH activity may result from a mutation in the PAH enzyme, for example, a mutation in the catalytic domain of PAH or one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N,

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A373T, V388M E390G, A395P, P407S, and Y414C; or subjects that are pregnant females, females of child-bearing age that are contemplating pregnancy, or infants between 0 and 3 years of age, or 0-2, 0-1.5 or 0-1; or subjects diagnosed as unresponsive within 24 hours to a single-dose BH4 loading test or a multiple dose loading test, such as a 4-dose or 7-day loading test. Exemplary patient populations and exemplary BH4 loading tests are described in Int'l. Publication No. WO 2005/049000, incorporated herein by reference in its entirety.

U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment as described herein.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg  $\times$  5 kg body weight) to 3000 mg/day (30 mg/kg  $\times$  100 kg body weight). While continuous, daily administration is contemplated, for HPA it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again. Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data.

In preferred embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

The invention further contemplates that stable formulations of the invention may be used for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from co-treatment with folates, including folate precursors, folic acids, or folate derivatives.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular

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arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (narrowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, such as hypertension, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, peripheral circulation disorder, coronary arterio- stenosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arterio- sclerotic heart diseases or the like so far as they are associated with insulin resistance. It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, espe-

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cially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

#### V. Combination Therapy

Certain methods of the invention involve the combined use of the stable formulations of the invention and one or more other therapeutic agents.

In such combination therapy, administration of the stable formulations of the invention may be concurrent with or may precede or follow the administration of the second therapeutic agent, e.g. by intervals ranging from minutes to hours, so long as both agents are able to exert their therapeutic effect at overlapping time periods. Thus, the invention contemplates the stable formulations of the invention for use with a second therapeutic agent. The invention also contemplates use of a second therapeutic agent in preparation of a medicament for administration with the stable tetrahydrobiopterin, precursor, derivative or analog formulations of the invention.

Tetrahydrobiopterin therapy may be combined with dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. For example, one could administer to the subject the BH4 composition and a low-phenylalanine medical protein composition in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, with a delay time of only about 1 hour being most preferred. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in

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tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Genet. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4)243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

## VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Preparation of Stabilized Crystallized Form of BH4

Int'l. Publication No. WO 2005/065018, incorporated herein by reference in its entirety, describes X ray and Raman spectra studies to characterize the polymorphs, including hydrates or solvates, of BH4, as well as exemplary crystallization conditions under which the polymorphs can be prepared. Int'l.

Publication No. WO 2005/049000, incorporated herein by reference in its entirety, describes various patient populations for which BH4 treatment is suitable and describes methods for treating such subjects with BH4. Int'l Publication No. WO2005/049614 incorporated herein by reference in its entirety, describe methods of synthesizing BH4.

The references cited herein throughout, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.

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### Example 2

#### Stable Tablet Formulation of Tetrahydrobiopterin

Three tablet formulations were prepared by mixing the ingredients shown in Table I as described in detail below.

TABLE I

Ingredient	Formulation I (wt %)	Formulation II (wt %)	Formulation III (wt %)
6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt, polymorph B (Active Ingredient)	33.33	33.33	33.33
D-Mannitol (Taste Masking)	57.56	57.56	57.56
Dibasic Calcium Phosphate, Anhydrous (Binder)	2.18	2.18	2.18
Hydroxypropyl Cellulose (Disintegrant)	3.63	4.5	
Polyvinylpyrrolidone (Disintegrant)	0.87		4.50
Ascorbic acid (Stabilizer)	1.67	1.67	1.67
Riboflavin (Coloring Agent)	0.01	0.01	0.01
Sodium Stearyl Fumarate (Lubricant)	0.75	0.75	0.75

For each formulation in Table I, twelve kilogram batches were prepared by first charging 4 kg of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt (SAPROPTERIN Hydrochloride, available from Daiichi Suntory Pharma Co., Ltd., Japan to a blender and blending the BH4 for 10 minutes at 25 revolutions per minute (RPM). Then 6.91 kg of D-Mannitol (PEARLITOL, available from Roquette America, Inc., Keokuk, Iowa) was added to the blender and the mixture was allowed to blend for an additional 10 minutes at 25 RPM. Then 260 grams of Anhydrous Dibasic Calcium Phosphate (available from Mallinckrodt Baker, Inc., Phillipsburg, N.J.) and (a) in Formulation I, 436 grams of Hydroxypropyl Cellulose and 104 grams were added to the blender, (b) in Formulation II 540 grams of Hydroxypropyl Cellulose was added to the blender; (c) in Formulation III, 540 grams of Polyvinylpyrrolidone (KOLLIDON CL, available from BASF Corporation, Florham Park, N.J.) were added to the blender, and the mixture was allowed to blend for an additional 10 minutes at 25 RPM. To the blender 200 grams of Ascorbic Acid and 120 grams of Riboflavin were added to the blender and the mixture was allowed to blend for 3 minutes at 25 RPM. The Sodium Stearyl Fumarate lubricant (PRUV, available from Penwest Pharmaceuticals Co., Danbury, Conn.) was filtered through a 25 mesh stainless steel screen and into a bag, and the blender was then charged with 9 kg of the screened Sodium Stearyl Fumarate, and the resulting mixture was allowed to blend for 5 minutes at 25 RPM.

The blended mixture of each formulation were then removed from the blender, and three samples of each formulation were collected for the preparation of a 150 mg, a 300 mg, and a 600 mg tablets. For each formulation, the tablet samples (150 mg, 300 mg, and 600 mg) were placed in a tablet press (available from Jenn-Chiang Mahinery Co., Ltd., Taiwan, R.O.C.) wherein the parameters of the tablet press were set to provide tablets with a thickness in the range of 4.5 to 5.5 millimeters, and a target hardness of 7 KP.



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The resulting tablets were then analyzed to determine the stability of the formulations. The stability of the formulations was studied for a change in appearance over time by a visual inspection at different intervals, for disintegration of the formulation utilizing the United States Pharmacopeia recommendations no. 701, and for a chemical change by assaying the components of the formulations. The results of the stability tests are summarized below in Table II.

TABLE II

Form.	Test	Initial	2 weeks	4 weeks	8 weeks
I	Appearance	Off white, round Tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablets	Dark yellow spots (diameter 1-2 mm) on the off white tablet
	Disintegration	5 min 20 sec	5 min 40 sec	8 min 4 sec	—
	Chemical Assay	97.10%	97.90%	98.2	100.7
II	Appearance	Off white round Tablets	Broken chip on some tablets. Light yellow spots (diameter 1-2 mm) on the off white round tablets.	Broken chip on some tablets. Light yellow spots (diameter 1-2 mm) on the off white round tablets	Broken chip on some tablets. Light brown spots (diameter 1-2 mm) on the dark brown tablets
	Disintegration	4 min 10 sec	4 min 38 sec	6 min 52 sec	—
	Chemical Assay	102.70%	100.00%	100.2	97.4
III	Appearance	Color is off white	Rough surface, and color is light yellow	Rough surface, and color is light yellow	Rough surface, and color is yellow
	Disintegration	1 min 52 sec	35 sec	58 sec	—
	Chemical Assay	100.20%	102.90%	97.4	99.8

The stability tests show that tablet Formulation III is more stable than the other formulations of BH4. Each of the pharmaceutical preparations are useful formulations for the delivery of BH4. Formulation III exhibited better stability than Formulations I and II. Thus, in one preferred embodiment, the stabilized tablet formulation comprises an optimal disintegration agent, for example, croscopolone or a disintegration agent more similar to polyvinylpyrrolidone than hydroxypropylcellulose. The preferred formulation is Formulation III. Other suitable tablet formulations may include at least ascorbic acid at a concentration of at least 0.01% weight, or at least 0.05% weight or at least 0.1% weight.

## Example 3

Three hundred mg tablets containing 100 mg tetrahydrobiopterin are prepared using the desired initial amount of polymorph B and mixing with other ingredients in the relative amounts shown below in Table III using the following dry tableting process. Tablets containing other desired amounts of tetrahydrobiopterin can be prepared in a similar manner.

The 6R-L-erythro-5,6,7,8-tetrahydrobiopterin and D-Mannitol were hand screened with a 20 mesh screen (designed to filter out particles greater than ~850  $\mu$ m in size) and placed in a blender. The mixture was blended for 10 minutes at 21 RPM. Next, the Anhydrous Dibasic Calcium Phosphate and CROSPVIDONE were hand screened with a 20 mesh screen and blended with the BH4 and D-Mannitol for 10

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minutes at 21 RPM. The Ascorbic Acid and Riboflavin were added to the blender after hand screening with a 20 mesh screen, and the resulting mixture blended for 10 minutes at 21 RPM. Next, the Sodium Stearyl Fumarate was added to the blender after hand screening with a 40 mesh screen, and blended for 5 minutes at 21 RPM. The blended mixture was then discharged into a bag and tested for uniformity, before being pressed into tablets.

TABLE III

Ingredient	(wt %)	(mg)
6R-L-erythro-5,6,7,8-tetrahydrobiopterin dihydrochloride salt (Active Ingredient)	33.33	100.0
D-Mannitol (Taste Masking)	57.56	172.68
Dibasic Calcium Phosphate, Anhydrous (Binder)	2.18	6.54
CROSPVIDONE (Disintegration Agent)	4.50	13.50
Ascorbic acid (Acidic Antioxidant)	1.67	5.00
Riboflavin (Coloring Agent)	0.01	0.03
Stearyl Fumarate (Lubricant)	0.75	2.25

The tablets were packaged either in foil blister-packs, or in HDPE bottles in quantities of 45-tablets per bottle. Each type of packaged tablets were divided into two batches. One batch was stored at room temperature,  $25 \pm 2^\circ \text{C}$ ., and  $60 \pm 5\%$  relative humidity. The other batch was stored under accelerated testing conditions, at  $40 \pm 2^\circ \text{C}$ . and  $75 \pm 5\%$  relative humidity. At regular intervals, tablets were removed from storage and tested for retention of the active pharmaceutical ingredient, 6R-L-erythro-5,6,7,8-tetrahydrobiopterin. Exemplary results for the 300 mg tablets are shown below in Tables IV, V, VI and VII. After six months storage under room temperature or accelerated testing conditions, each of the four batches showed retention of at least 99% of the original amount of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin by HPLC assay, loss on drying of less than 1%, and rapid disintegration in 3 minutes or less.

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TABLE IV

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, FOIL BLISTER-PACK) STORAGE CONDITIONS: 25 ± 2° C./60 ± 5% RH						
Test/Assay	Stability Specification	Time Point (months)				
		0	1	3	6	9
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	Conforms	Conforms
Loss on Drying	Report Result	0.3%	0.3%	0.9%	0.7%	0.9%
Disintegration	≤3 minutes	1 min. 30 sec.	2 min. 7 sec.	2 min. 2 sec.	2 min. 35 sec.	1 min. 48 sec.
HPLC Assay	90-110%	101%	100%	100%	100%	101%
HPLC for Related Substances	≤0.1% individual unidentified	0.05%	0.04% (RRT 1.28)	0.05% (RRT 0.72) 0.08% (RRT 0.83) 0.05% (RRT 1.32)	0.09% (RRT 0.81) 0.04% (RRT 0.83) 0.06% (RRT 1.24)	0.03% (RRT 0.69) 0.01% (RRT 0.73) 0.07% (RRT 0.81) 0.03% (RRT 0.83) 0.02% (RRT 1.21) 0.04% (RRT 1.44)
	≤0.5% total unidentified	0.05%	0.04%	0.18%	0.22%	0.20%
	≤2.0% total related substances	0.05%	0.04%	0.18%	0.22%	0.20%

ND = None detected

TABLE V

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, FOIL BLISTER-PACK) STORAGE CONDITIONS: 40 ± 2° C./75 ± 5% RH					
Test/Assay	Stability Specification	Time Point (months)			
		0	1	3	6
Appearance by Visual Inspection	White to light yellow compressed tablets	Conforms	Conforms	Conforms	Conforms
Loss on Drying	Report Result	0.3%	0.3%	0.8%	0.7%
Disintegration	≤3 minutes	1 min. 30 sec.	2 min. 16 sec.	2 min. 47 sec.	2 min. 11 sec.
HPLC Assay	90-110%	101%	100%	101%	99%
HPLC for Related Substances	≤0.1% individual unidentified	0.05%	0.04% (RRT 1.28)	0.06% (RRT 0.64) 0.07% (RRT 0.72) 0.04% (RRT 0.83) 0.06% (RRT 1.31)	0.07% (RRT 0.74) 0.15% (RRT 0.83) 0.04% (RRT 0.83) 0.06% (RRT 1.24)
	≤0.5% total unidentified	0.05%	0.04%	0.23%	0.32%
	≤2.0% total related substances	0.05%	0.04%	0.23%	0.32%

ND = None detected

TABLE VI

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45 TABLET BOTTLES) STORAGE CONDITIONS: 25 ± 2° C./60 ± 5% RH				
Test/Assay	Stability Specification	Time point (months)		
		0	3	6
Appearance by Visual Inspection	White to light yellow compressed tablets	Con-forms	Con-forms	Con-forms

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TABLE VI-continued

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45 TABLET BOTTLES) STORAGE CONDITIONS: 25 ± 2° C./60 ± 5% RH				
Test/Assay	Stability Specification	Time point (months)		
		0	3	6
Loss on Drying	Report Result	0.7%	0.8%	1.1%

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TABLE VI-continued

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45 TABLET BOTTLES) STORAGE CONDITIONS: 25 ± 2° C./60 ± 5% RH				
Test/Assay	Stability Specification	Time point (months)		
		0	3	6
Disintegration	≤3 minutes	2 min 57 sec	2 min 3 sec	2 min 50 sec
HPLC Assay	90-110%	102%	NT	103%
HPLC for Related Substances	≤0.1% individual unidentified	0.06%	NT	0.08% (RRT 0.56) 0.04% (RRT 0.61) 0.05% (RRT 0.65) 0.04% (RRT 0.81)
	≤0.5% total unidentified	0.15%	NT	0.21%
	≤2.0% total related substances	0.15%	NT	0.21%
	Report Result (mg/tablet)	5	NT	5
	Total Aerobic Microbial Count ≤ 1000 cfu/g Test for <i>Escherichia coli</i> = absent	<100 cfu/g Absent	NR	NR

ND = None detected

NT = Not tested

NR = Not required

TABLE VII

SAPROPTERIN DIHYDROCHLORIDE DRUG PRODUCT (300-MG TABLET, 45-TABLET BOTTLES) STORAGE CONDITIONS: 40 ± 2° C./75 ± 5% RH				
Test/Assay	Stability Specification	Time Point (months)		
		0	3	6
Appearance by Visual Inspection	White to light yellow compressed tablets	Con- forms	Con- forms	Con- forms
Loss on Drying	Report Result	0.7%	1.1%	1.1%
Disintegration	≤3 minutes	2 min 57 sec	2 min 41 sec	2 min 48 sec
HPLC Assay	90-110%	102%	NT	101%
HPLC for Related Substances	≤0.1% individual unidentified	0.06%	NT	0.13% (RRT 0.56) 0.03% (RRT 0.61) 0.10% (RRT 0.65) 0.04% (RRT 0.81)
	≤0.5% total unidentified	0.15%	NT	0.30%
	≤2.0% total related substances	0.15%	NT	0.30%
	Report Result (mg/tablet)	5	NT	5

ND = None detected

NT = Not tested

NR = Not required

What is claimed is:

1. A formulation, comprising about 100 mg of crystal form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and about 5 mg of ascorbic acid in the form of a tablet.

2. The formulation of claim 1, further comprising crospovidone, dibasic calcium phosphate, D-mannitol, riboflavin and sodium stearyl fumarate.

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3. The formulation of claim 1, further comprising crospovidone.

4. The formulation of claim 1, further comprising dibasic calcium phosphate.

5. The formulation of claim 1, further comprising D-mannitol.

6. The formulation of claim 1, further comprising riboflavin.

7. The formulation of claim 1, further comprising sodium stearyl fumarate.

8. A formulation, comprising crystal form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and ascorbic acid in the form of a tablet, wherein the weight ratio of ascorbic acid to (6R)-L-erythro-tetrahydrobiopterin is about 1:5.5, about 1:6, about 1:6.5, about 1:7, about 1:7.5, about 1:8, about 1:8.5, about 1:9, about 1:9.5, about 1:10, about 1:10.5, about 1:11, about 1:11.5, about 1:12, about 1:12.5, about 1:13, about 1:13.5, about 1:14, about 1:14.5, about 1:15, about 1:15.5, about 1:16, about 1:16.5, about 1:17, about 1:17.5, about 1:18, about 1:18.5, about 1:19, about 1:19.5, about 1:20, about 1:20.5, about 1:21, about 1:21.5, about 1:22, about 1:22.5, about 1:23, about 1:23.5, about 1:24, about 1:24.5, about 1:25, about 1:25.5, about 1:26, about 1:26.5, about 1:27, about 1:27.5, about 1:28, about 1:28.5, about 1:29, or about 1:29.5.

9. The formulation of claim 8, further comprising crospovidone, dibasic calcium phosphate, D-mannitol, riboflavin and sodium stearyl fumarate.

10. The formulation of claim 8, further comprising crospovidone.

11. The formulation of claim 8, further comprising dibasic calcium phosphate.

12. The formulation of claim 8, further comprising D-mannitol.

13. The formulation of claim 8, further comprising riboflavin.

14. The formulation of claim 8, further comprising sodium stearyl fumarate.

15. The formulation of claim 8, wherein the weight ratio of ascorbic acid to (6R)-L-erythro-tetrahydrobiopterin is about 1:16.5, about 1:17, about 1:17.5, about 1:18, about 1:18.5, about 1:19, about 1:19.5, about 1:20, about 1:20.5, about 1:21, about 1:21.5, about 1:22, about 1:22.5, about 1:23, about 1:23.5, about 1:24, about 1:24.5, or about 1:25.

16. The formulation of claim 8, wherein the weight ratio of ascorbic acid to (6R)-L-erythro-tetrahydrobiopterin is about 1:19, about 1:19.5, or about 1:20.

17. A formulation, comprising crystal form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and ascorbic acid in the form of a tablet, wherein the weight ratio of ascorbic acid to (6R)-L-erythro-tetrahydrobiopterin is about 1:2, about 1:3, about 1:4, or about 1:5.

18. The formulation of claim 17, further comprising crospovidone.

19. The formulation of claim 17, further comprising dibasic calcium phosphate.

20. The formulation of claim 17, further comprising D-mannitol.

21. The formulation of claim 17, further comprising riboflavin.

22. The formulation of claim 17, further comprising sodium stearyl fumarate.

\* \* \* \* \*

# EXHIBIT E

US008067416B2

(12) **United States Patent**  
**Oppenheimer et al.**(10) **Patent No.:** **US 8,067,416 B2**  
(45) **Date of Patent:** **\*Nov. 29, 2011**(54) **METHODS AND COMPOSITIONS FOR THE TREATMENT OF METABOLIC DISORDERS**(75) Inventors: **Daniel I. Oppenheimer**, Castro Valley, CA (US); **Emil D. Kakkis**, Novato, CA (US); **Fredric D. Price**, Bedford, NY (US); **Alejandro Dorenbaum**, Mill Valley, CA (US); **Rudolf Moser**, Schaffhausen (CH); **Viola Groehn**, Dachsen (CH); **Thomas Egger**, Kempthal (CH); **Fritz Blatter**, Reinach (CH)(73) Assignees: **Merck Eprova AG**, Schaffhausen (CH); **Biomarin Pharmaceutical Inc.**, Novato, CA (US)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 252 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/508,209**(22) Filed: **Jul. 23, 2009**(65) **Prior Publication Data**

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**Related U.S. Application Data**

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(60) Provisional application No. 60/520,767, filed on Nov. 17, 2003.

(51) **Int. Cl.****A61K 31/495** (2006.01)**A61K 31/40** (2006.01)**A61K 31/445** (2006.01)**A01N 43/54** (2006.01)(52) **U.S. Cl.** ..... **514/249**; 514/338; 514/419; 514/258(58) **Field of Classification Search** ..... 514/249  
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

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*Primary Examiner* — Sreeni Padmanabhan*Assistant Examiner* — Timothy E Betton(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun LLP(57) **ABSTRACT**

The present invention is directed to a novel methods and compositions for the therapeutic intervention in hyperphenylalaninemia. More specifically, the specification describes methods and compositions for treating various types of phenylketonurias using compositions comprising BH4. Combination therapies of BH4 and other therapeutic regimens are contemplated.

**24 Claims, 20 Drawing Sheets**

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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form B

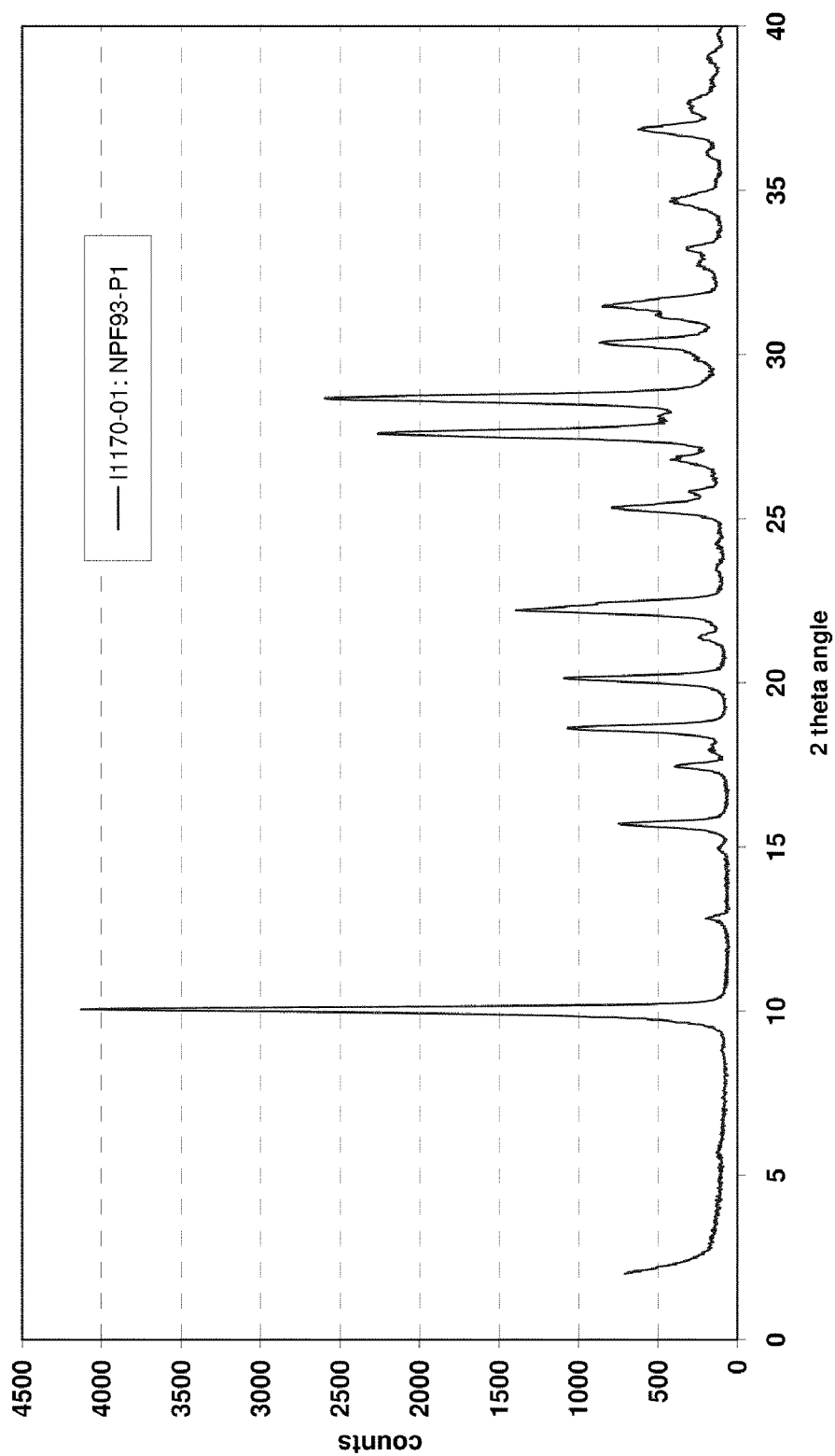


Figure 1

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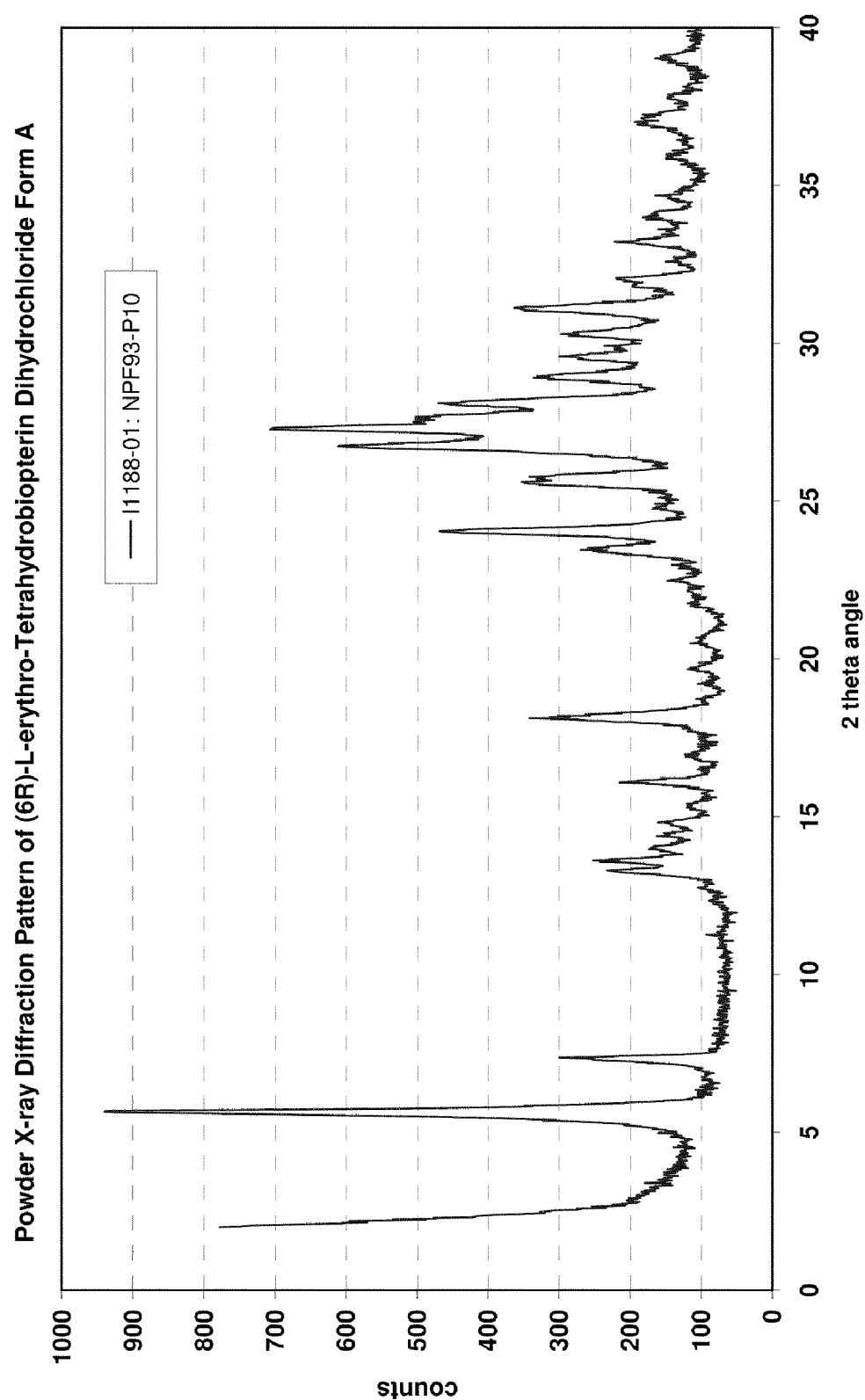


Figure 2



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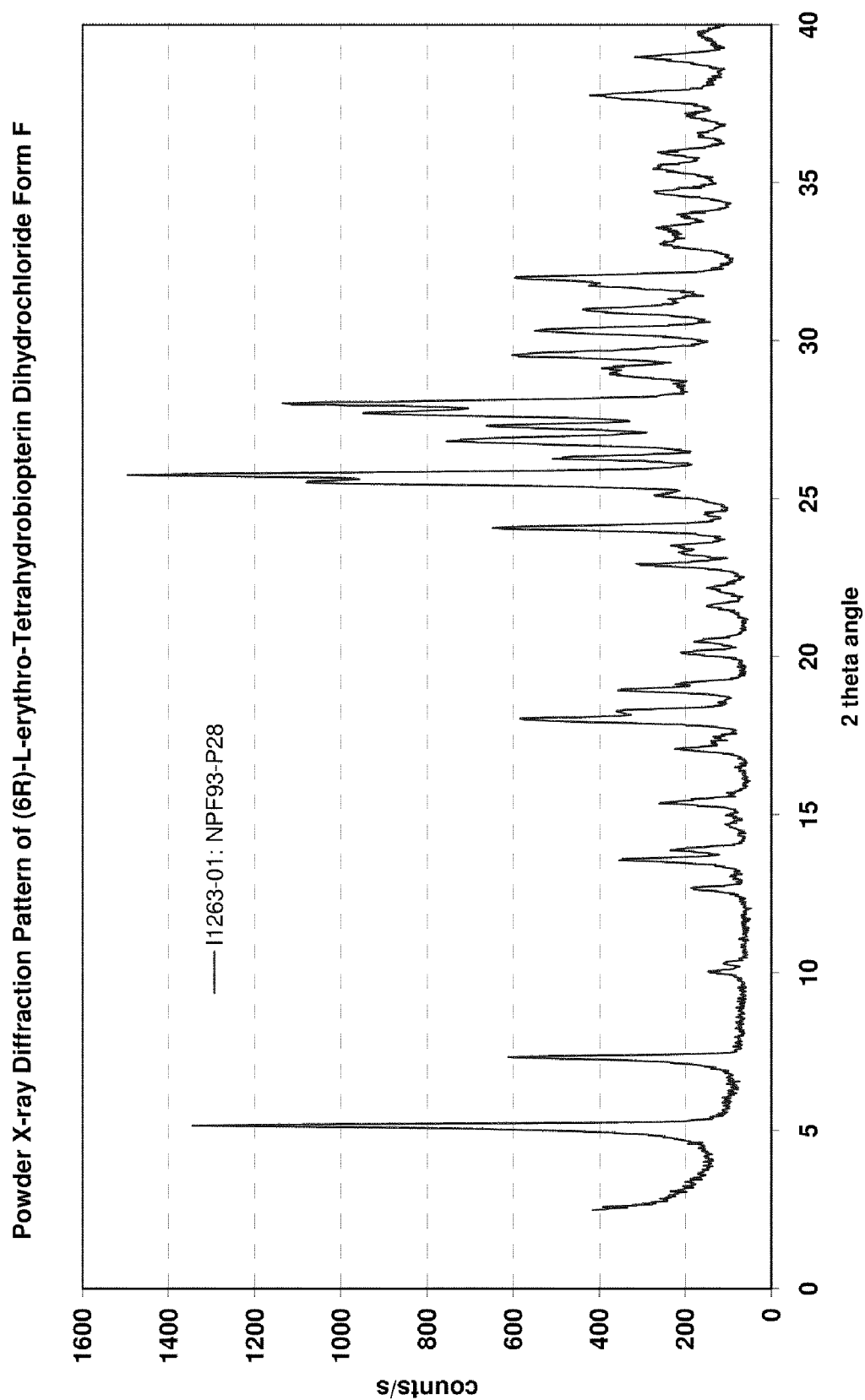


Figure 3



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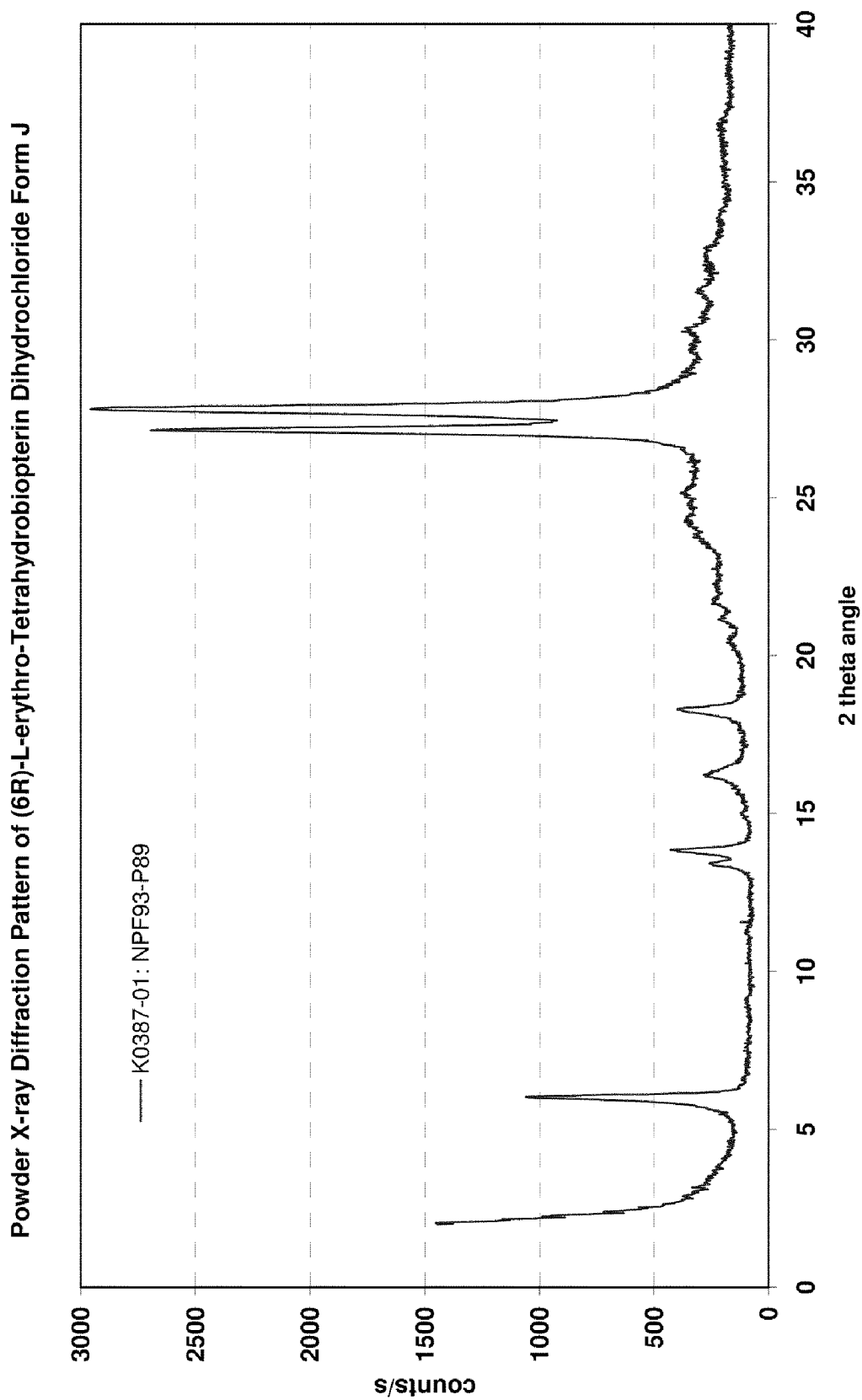


Figure 4

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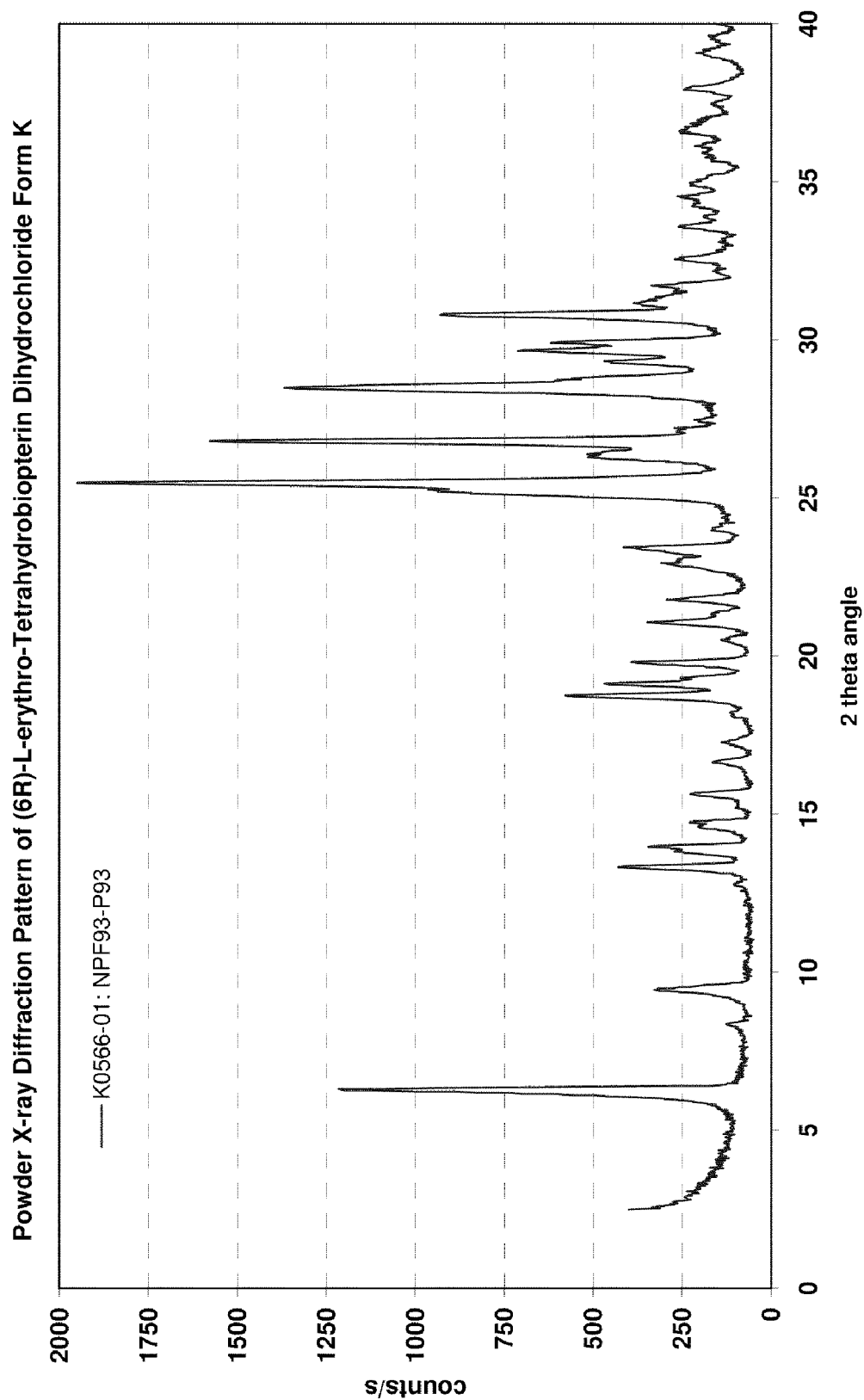


Figure 5

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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochlorid Form C

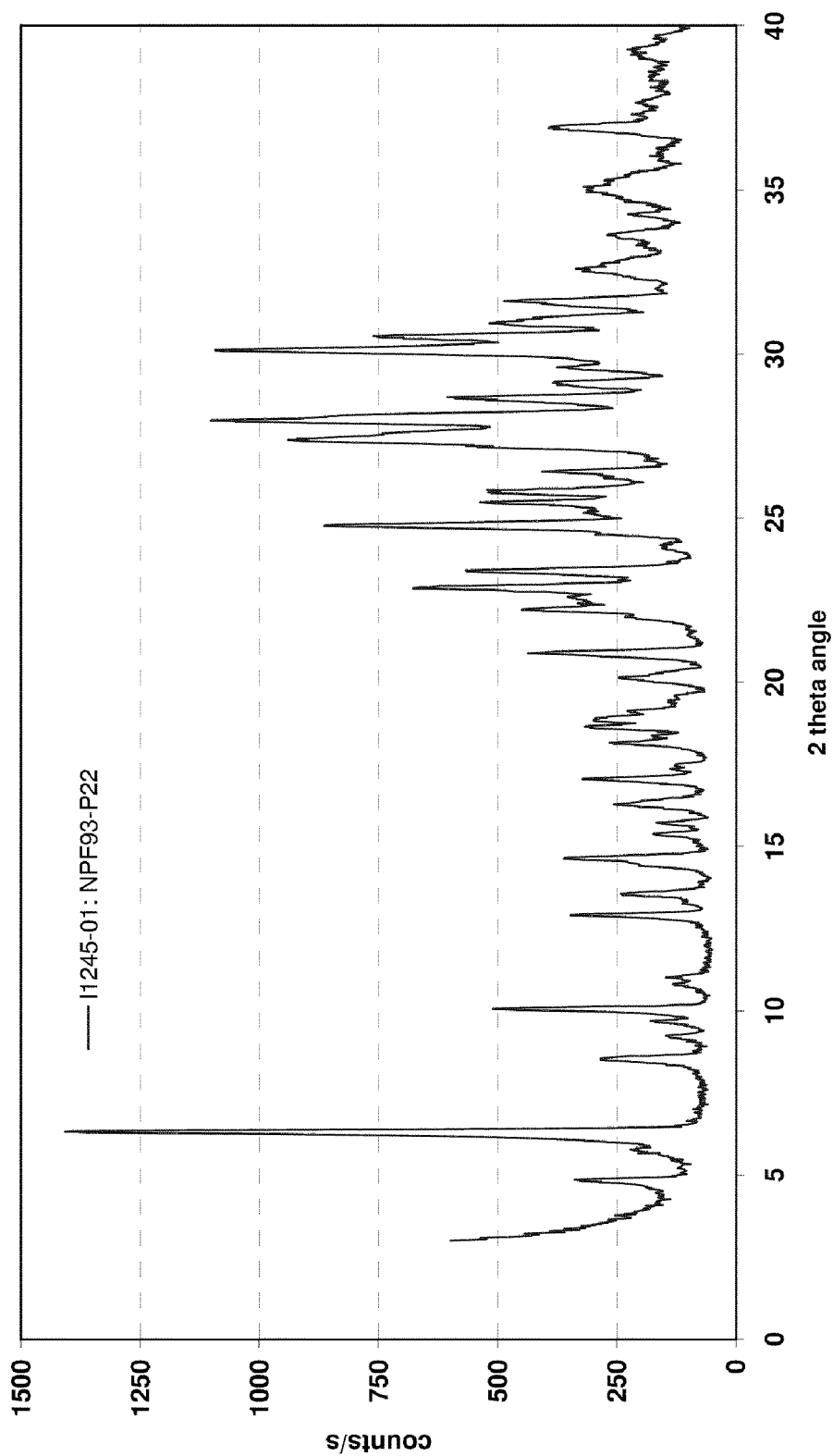


Figure 6

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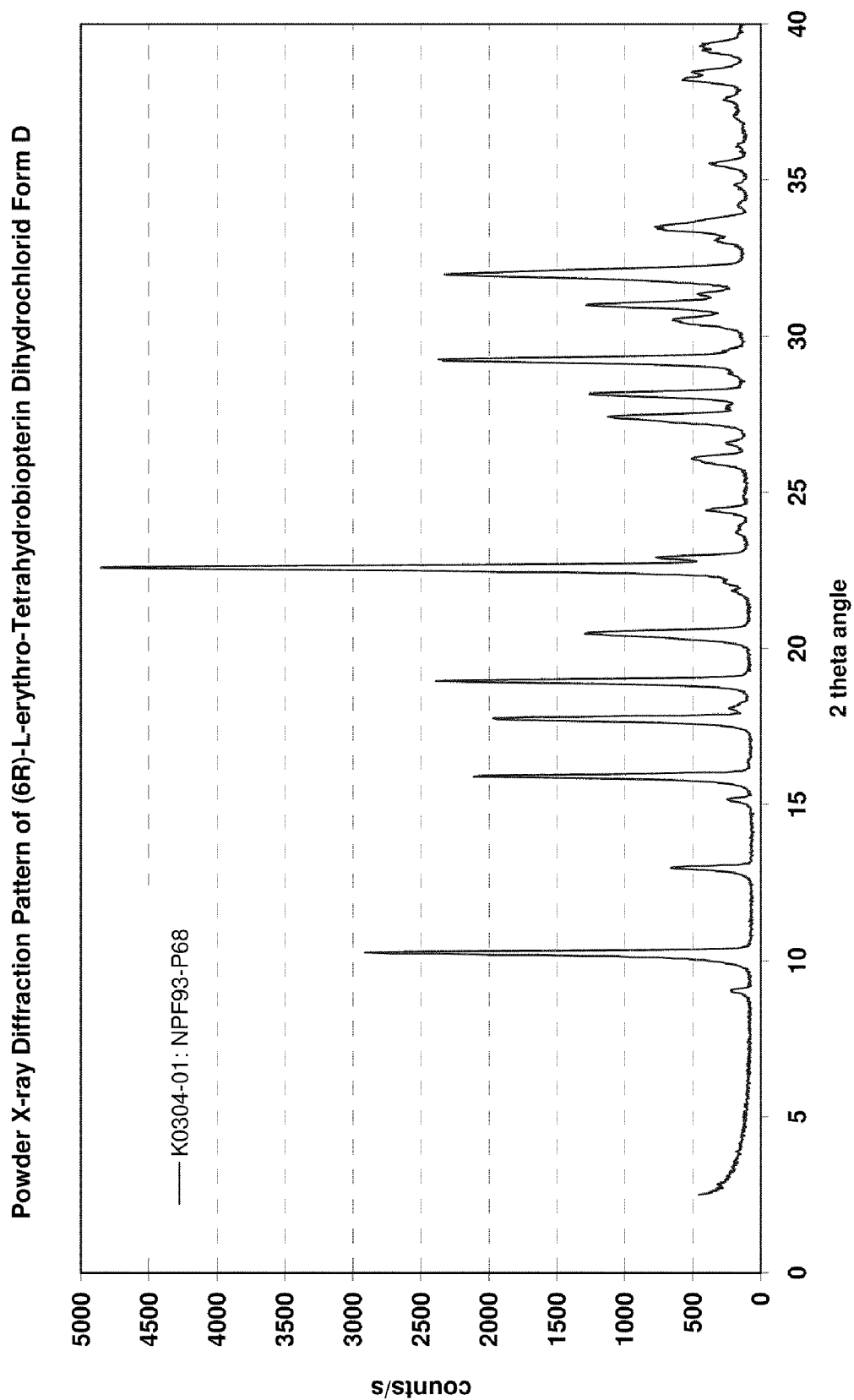


Figure 7

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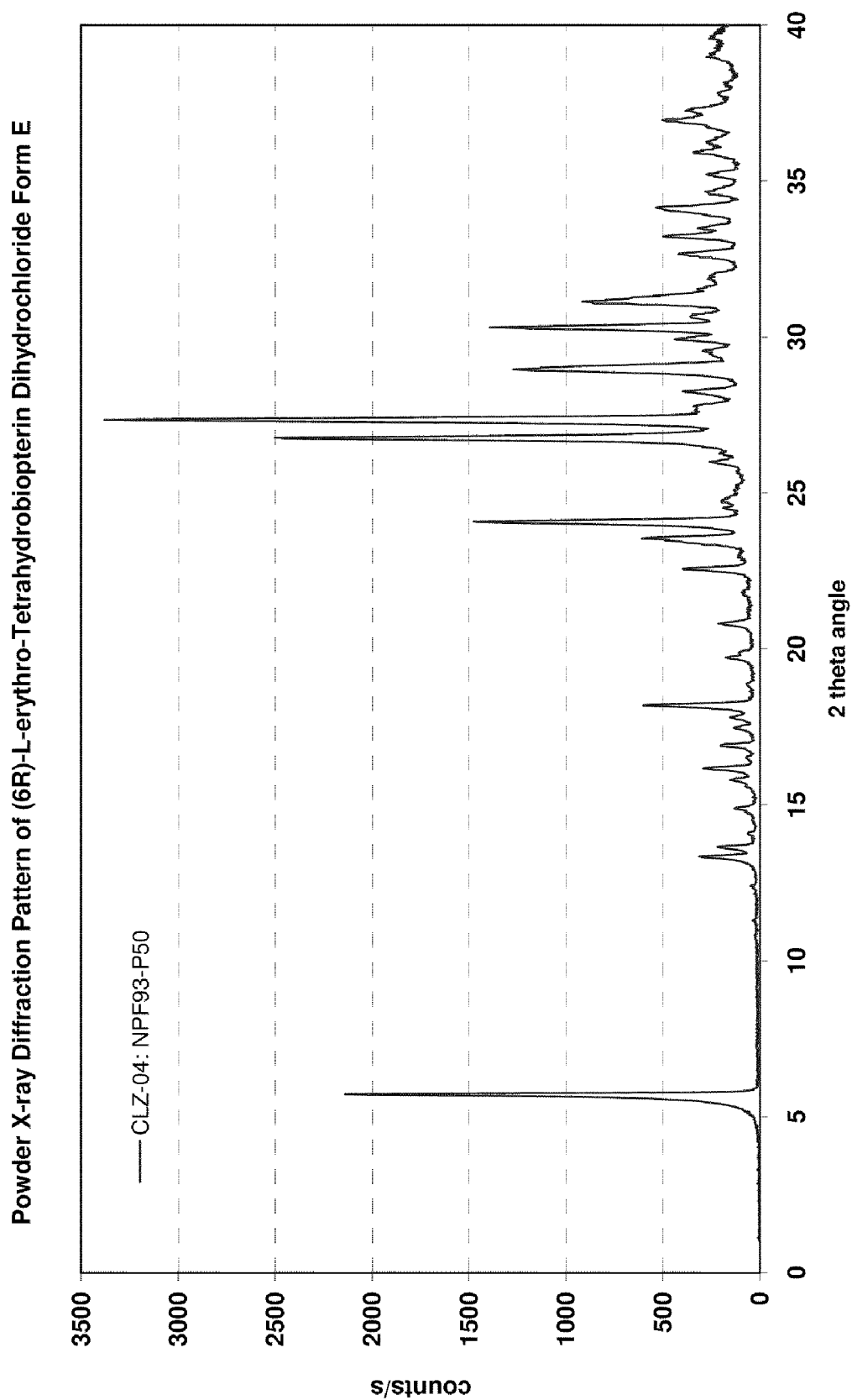


Figure 8

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Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form H

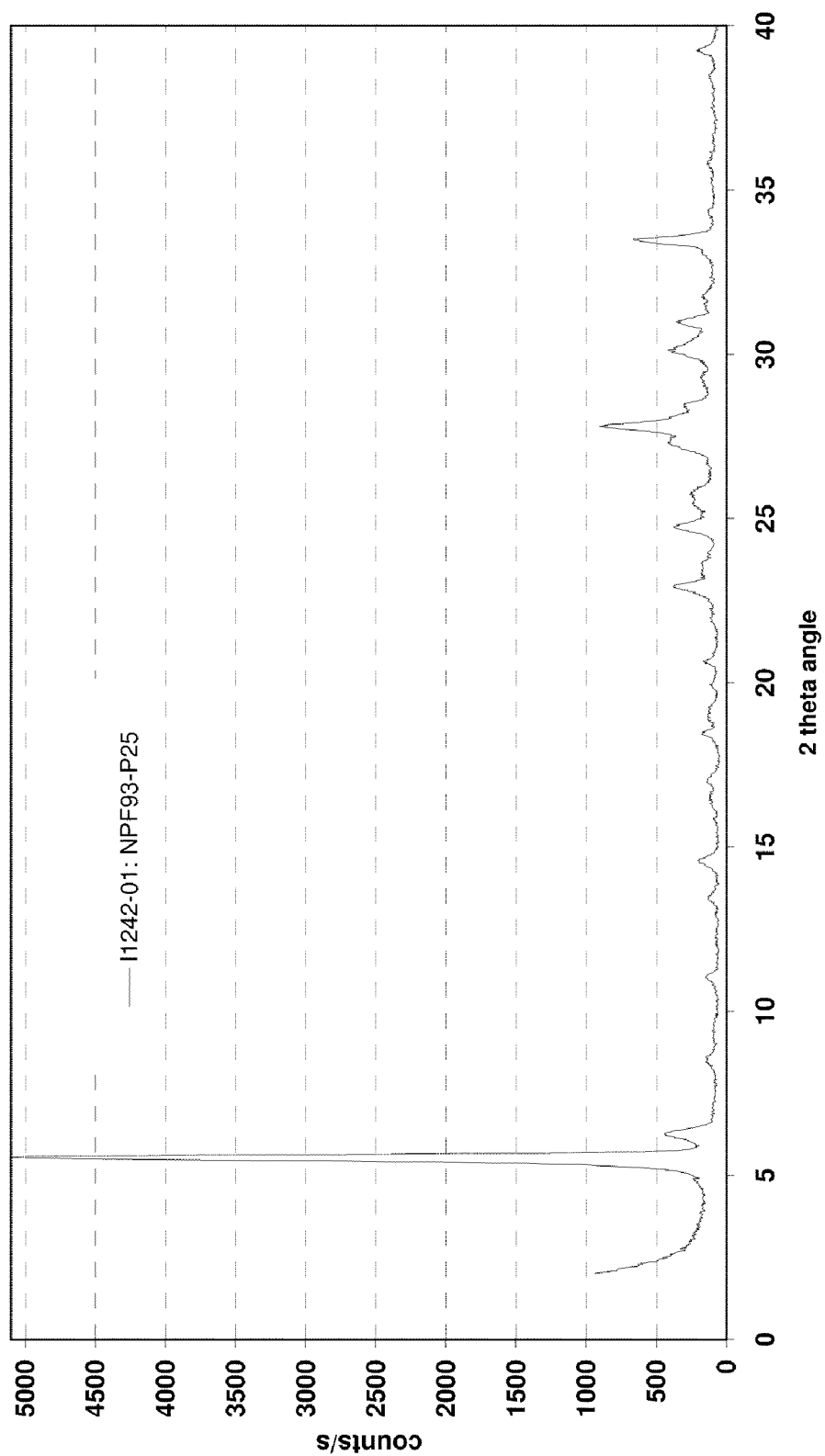


Figure 9



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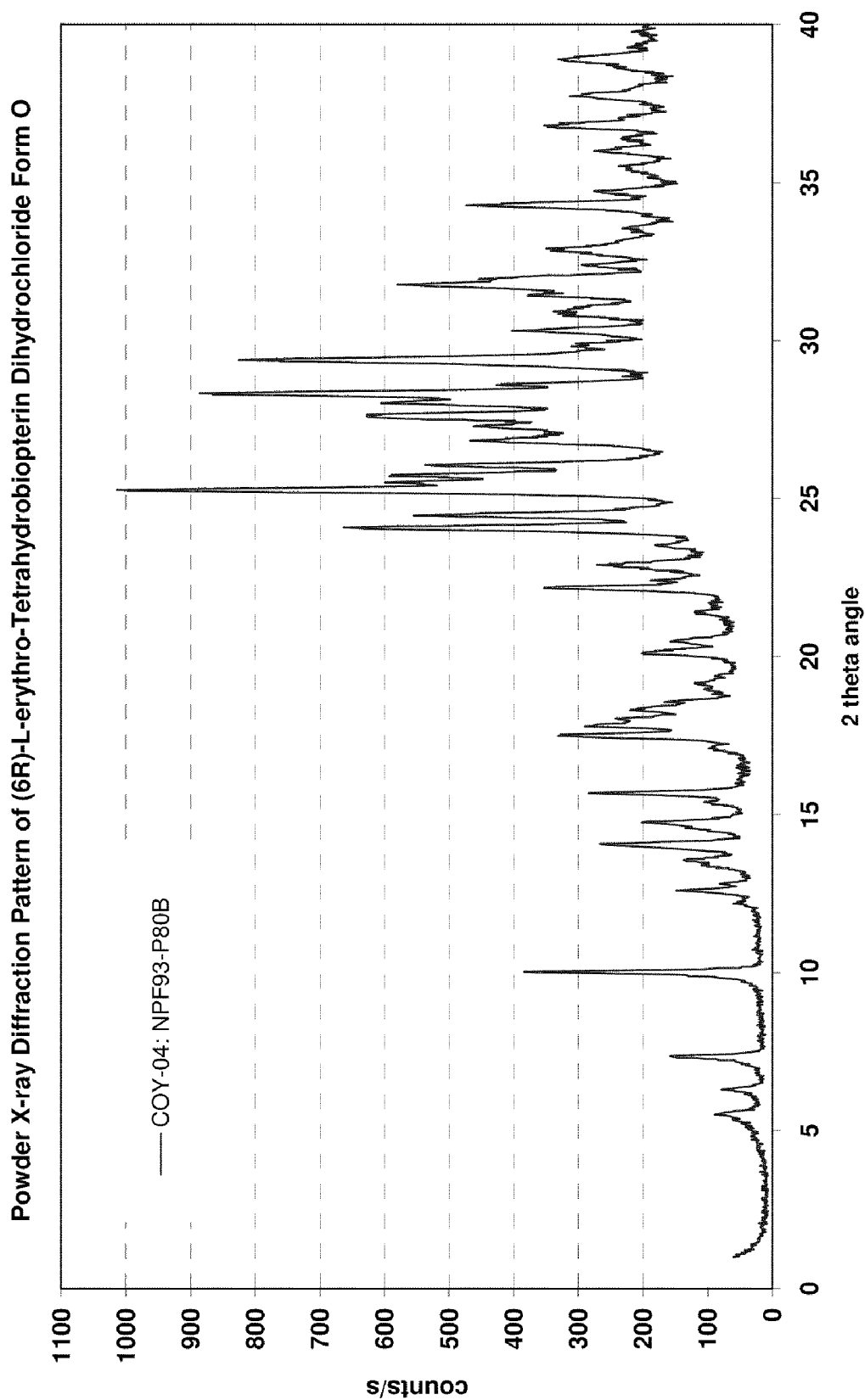


Figure 10

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form G

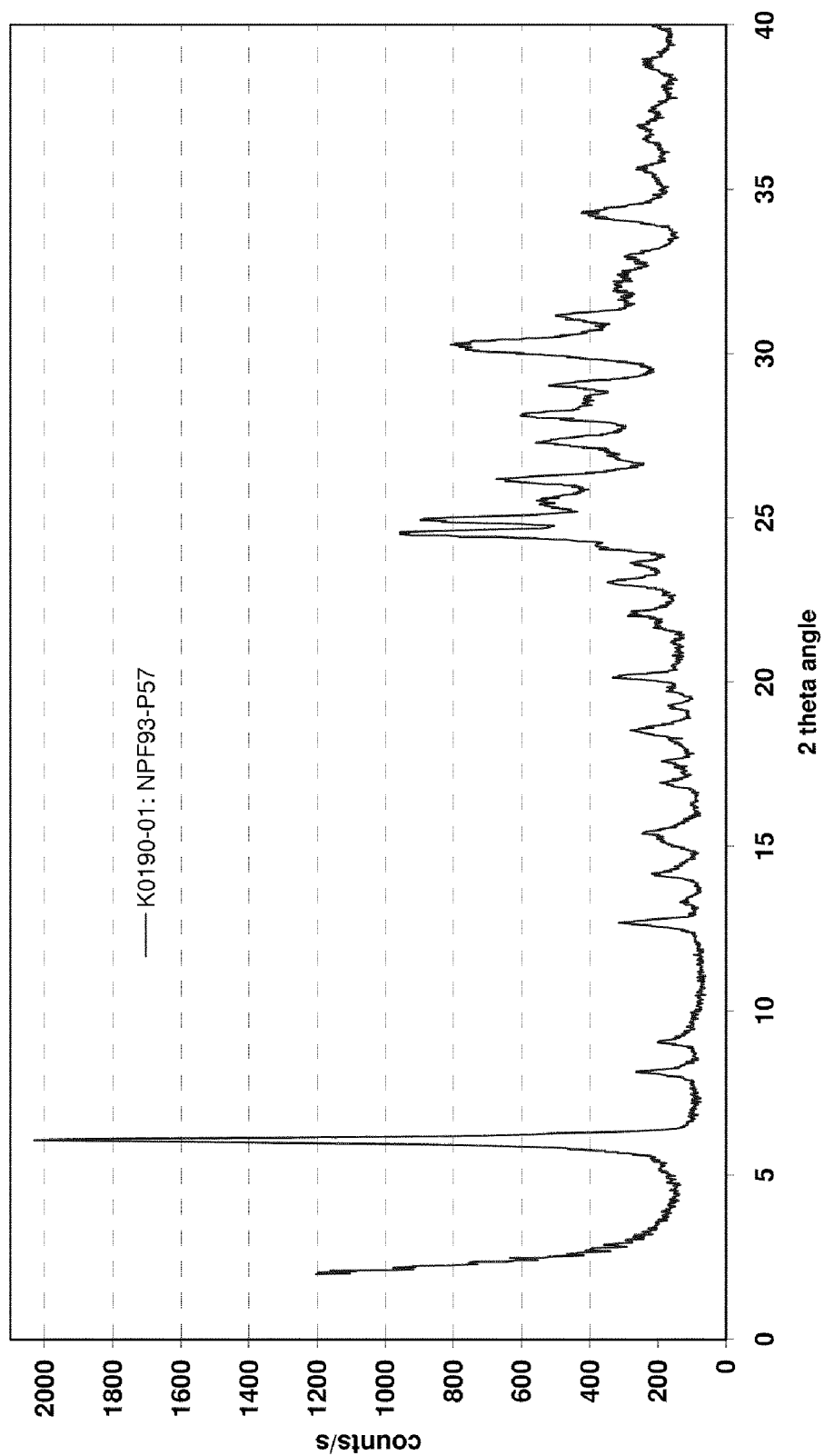


Figure 11

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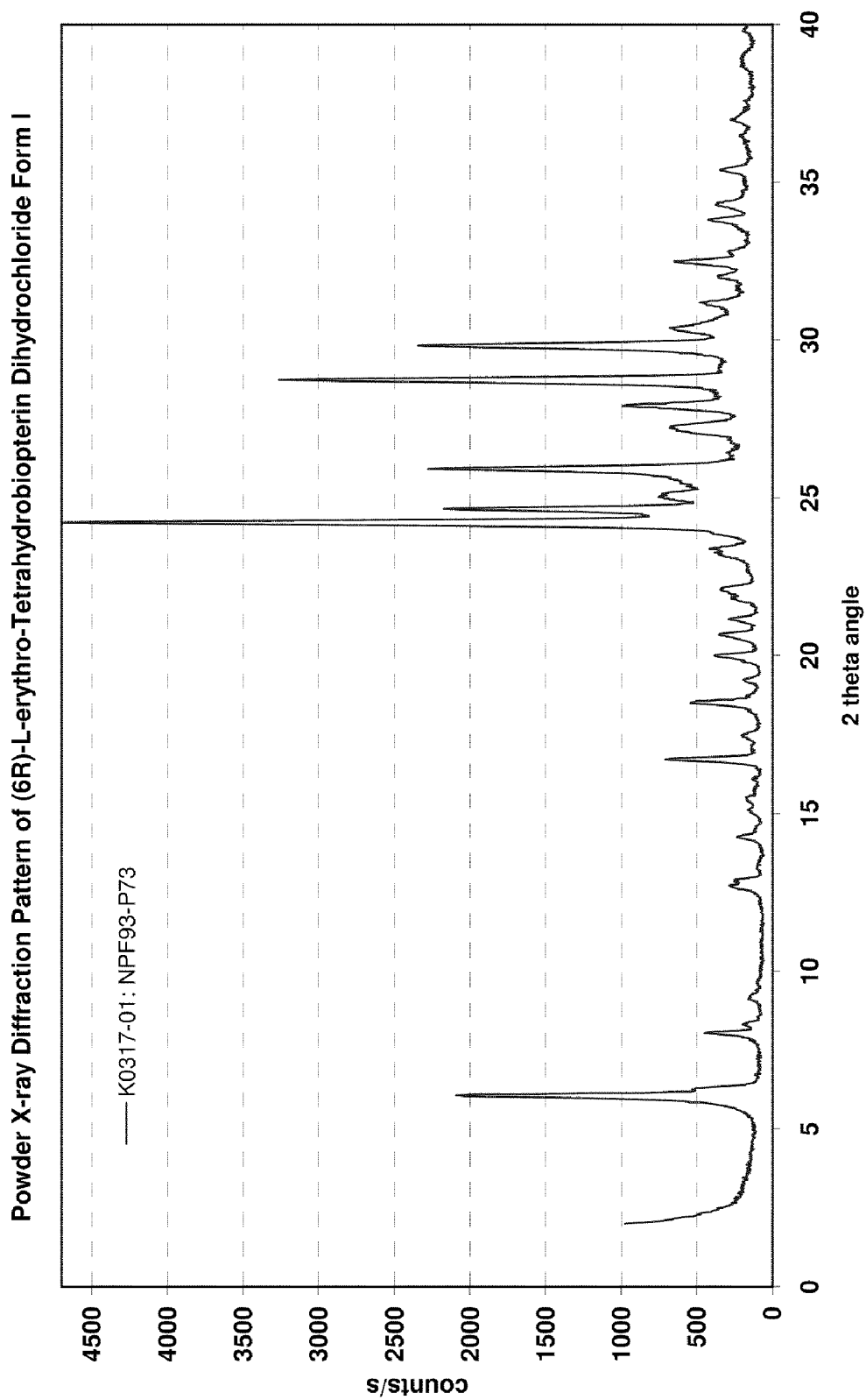


Figure 12

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form L

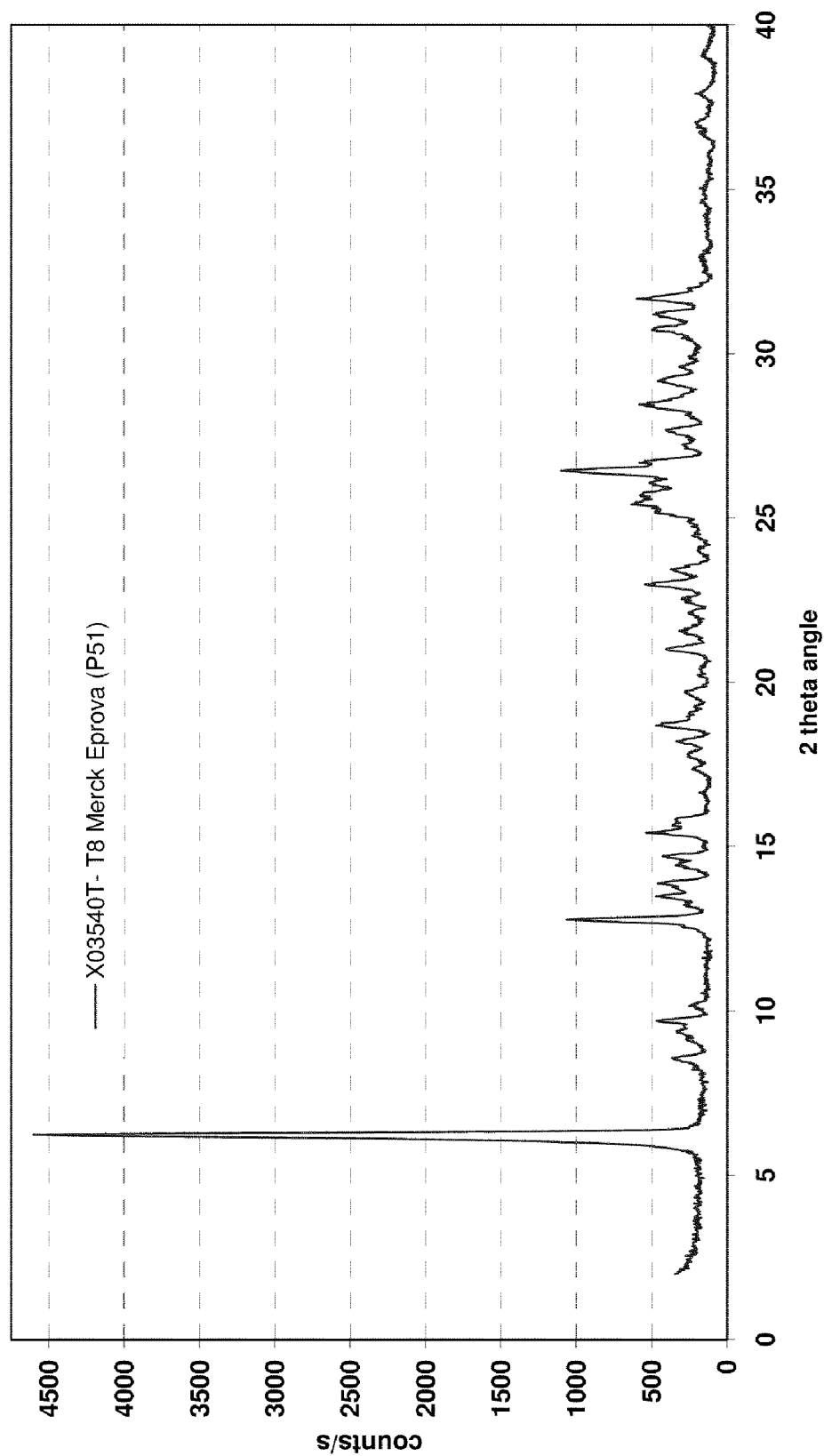


Figure 13

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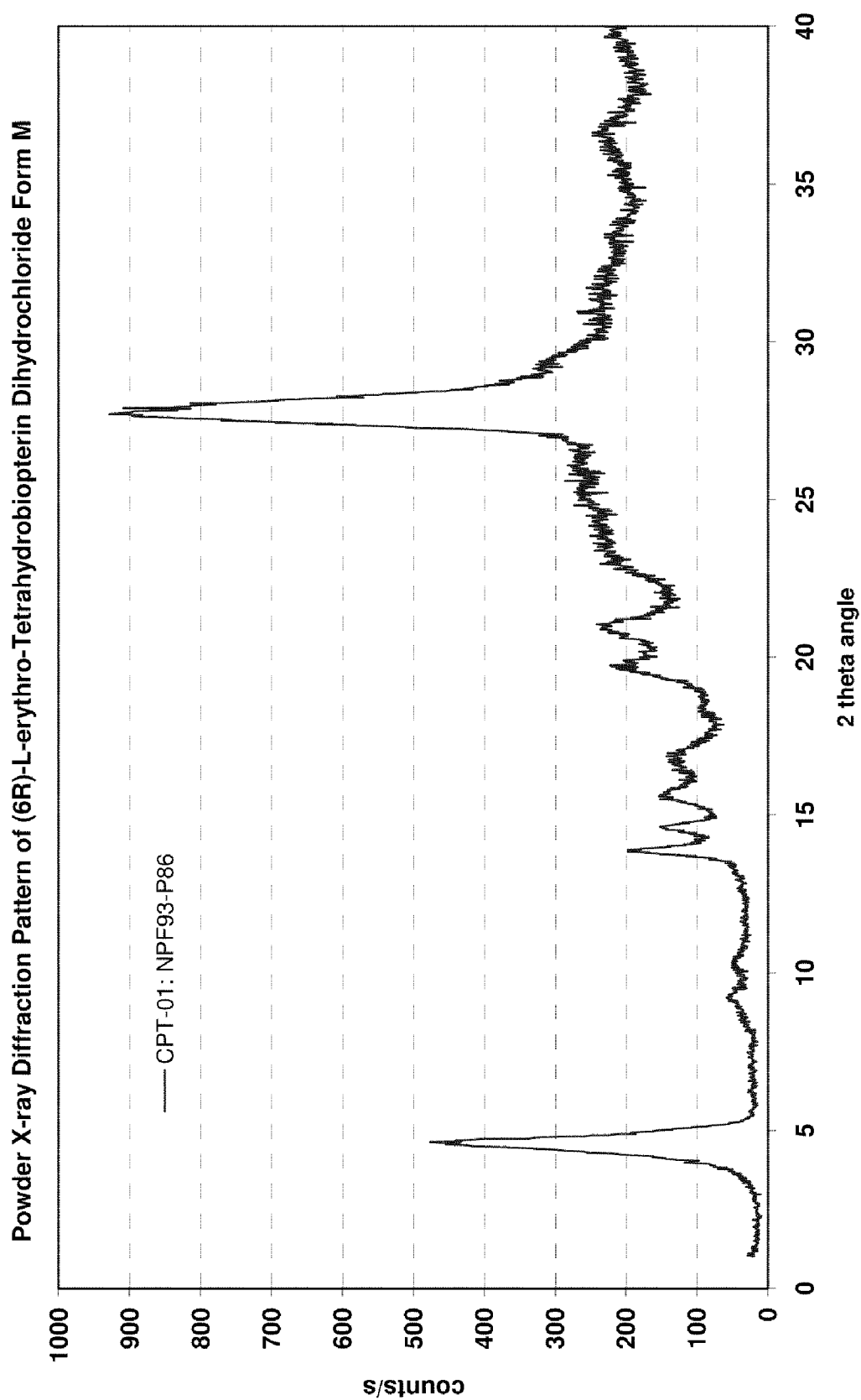


Figure 14

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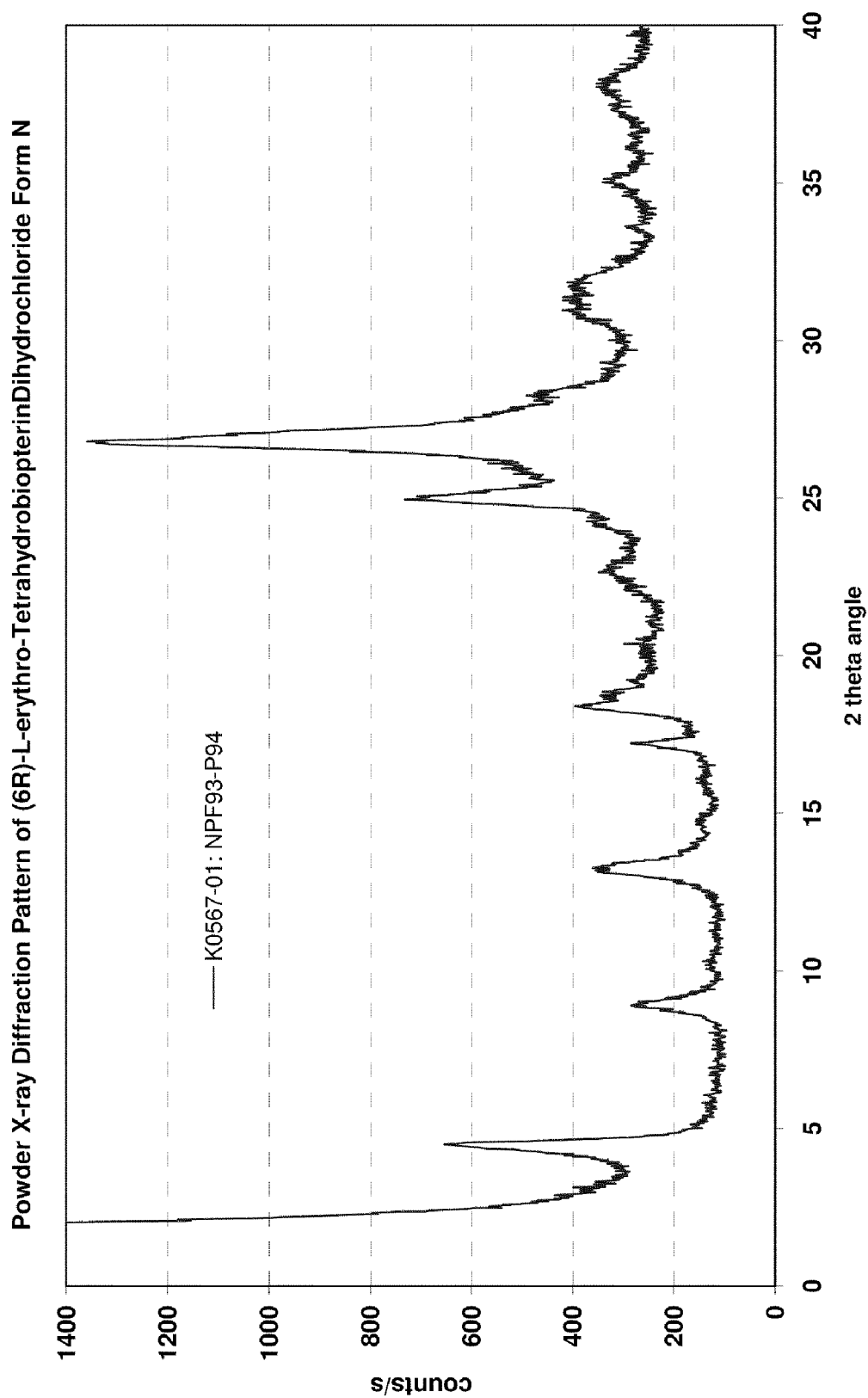


Figure 15



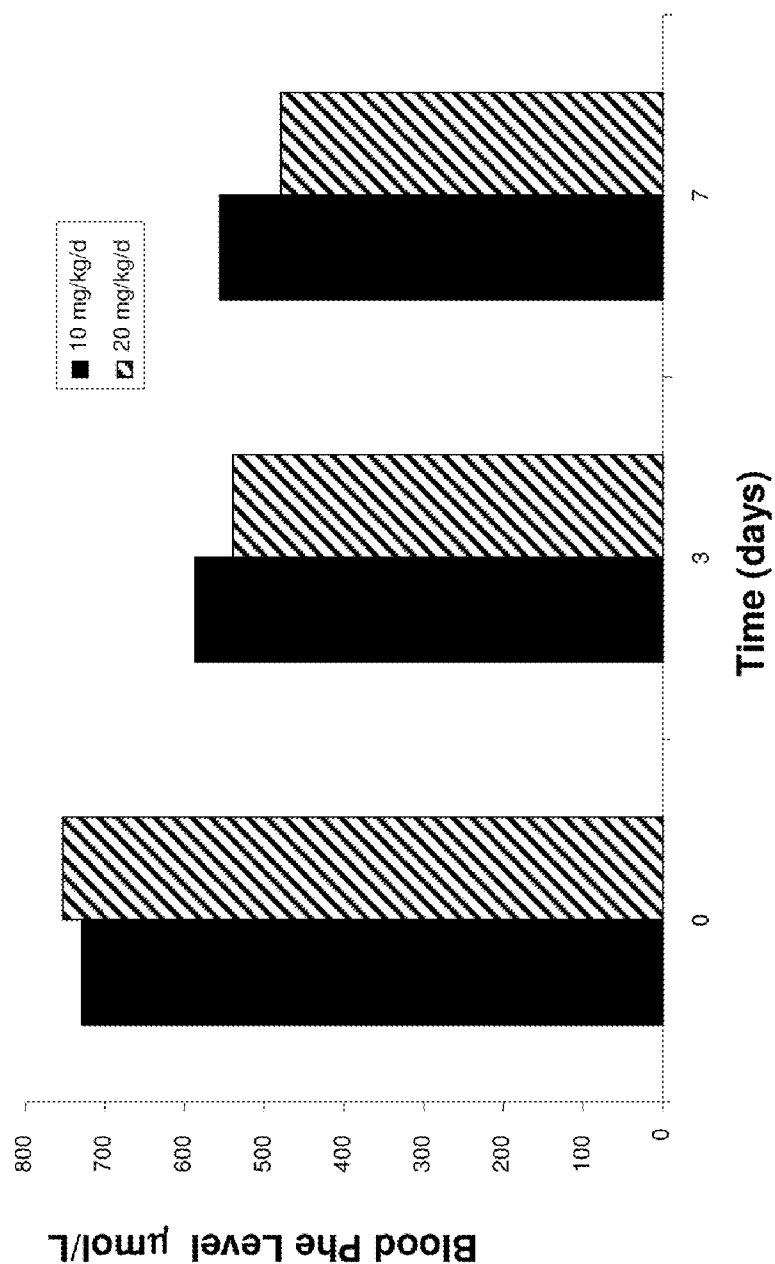
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**Mean Blood Phe Level 3 and 7 Days After  
Multiple Daily BH4 Doses of 10 and 20  
mg/kg in PKU Patients (N=20)**



**Figure 16**

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# Individual Blood Phe in 12 Adults with PKU on 10 mg/kg BH4 Daily

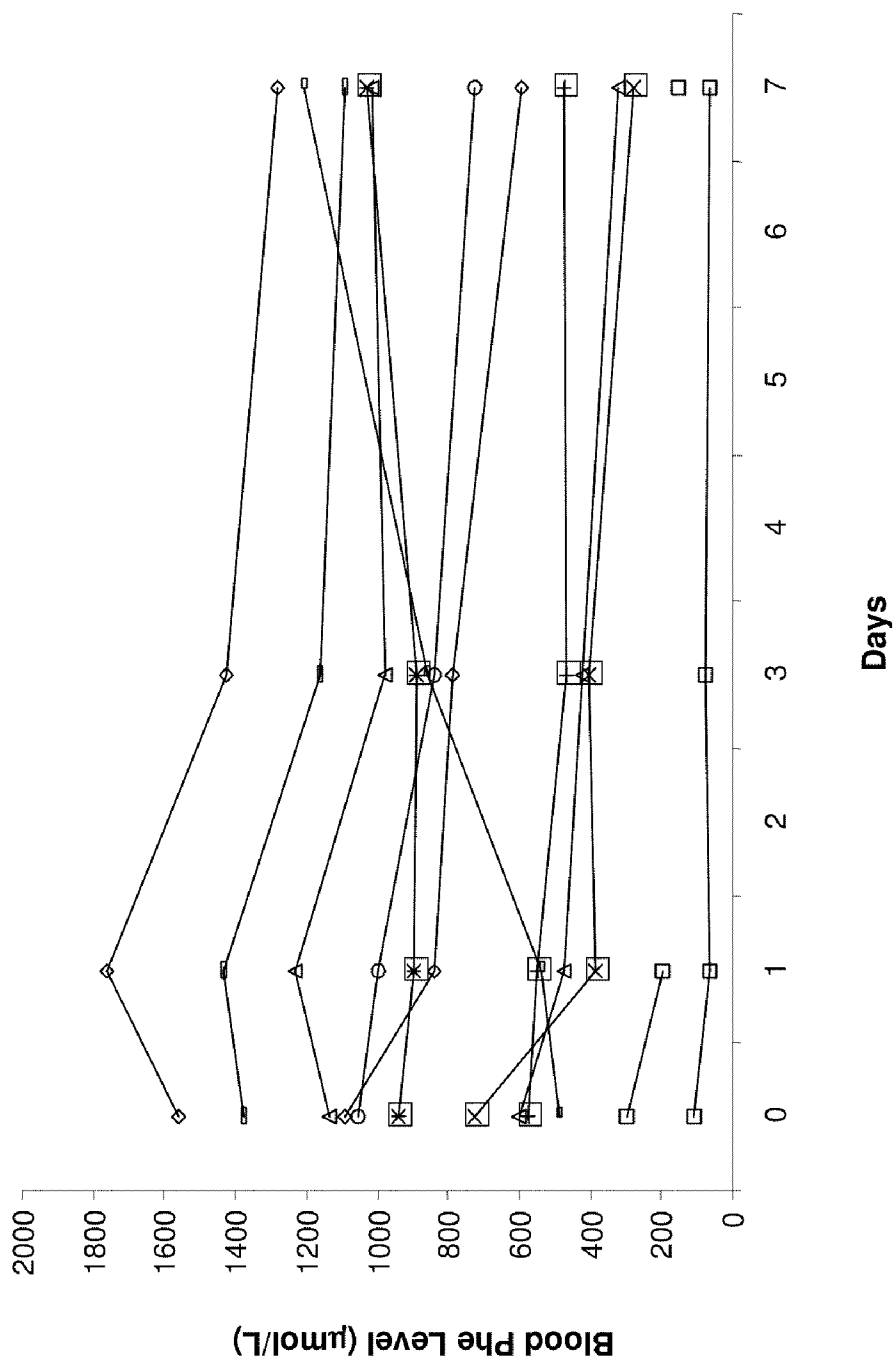


Figure 17

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# Individual Blood Phe in 12 Adults with PKU on 20 mg/kg BH4 Daily

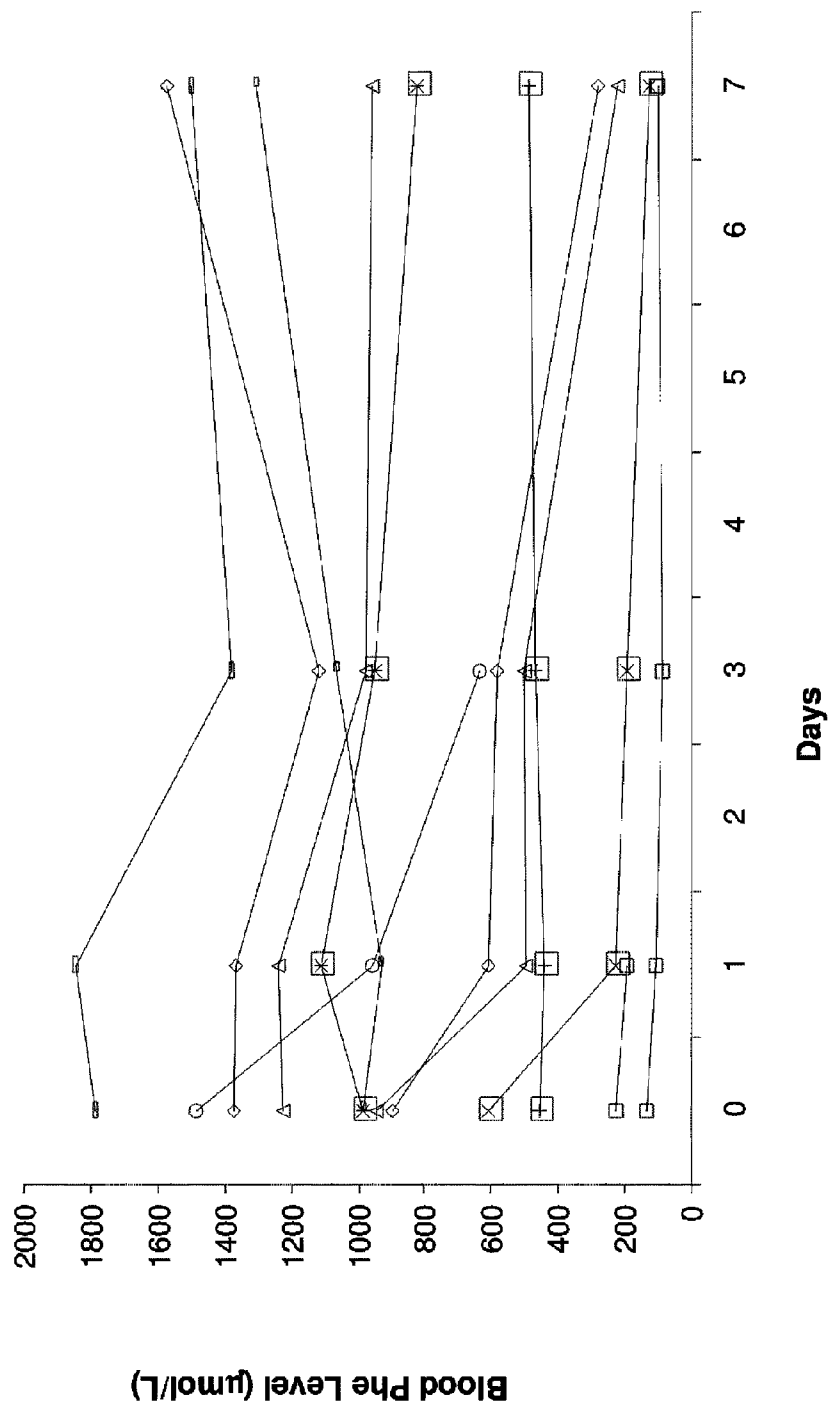


Figure 18

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# Individual Blood Phe in 8 Children with PKU on 10 mg/kg BH4 Daily

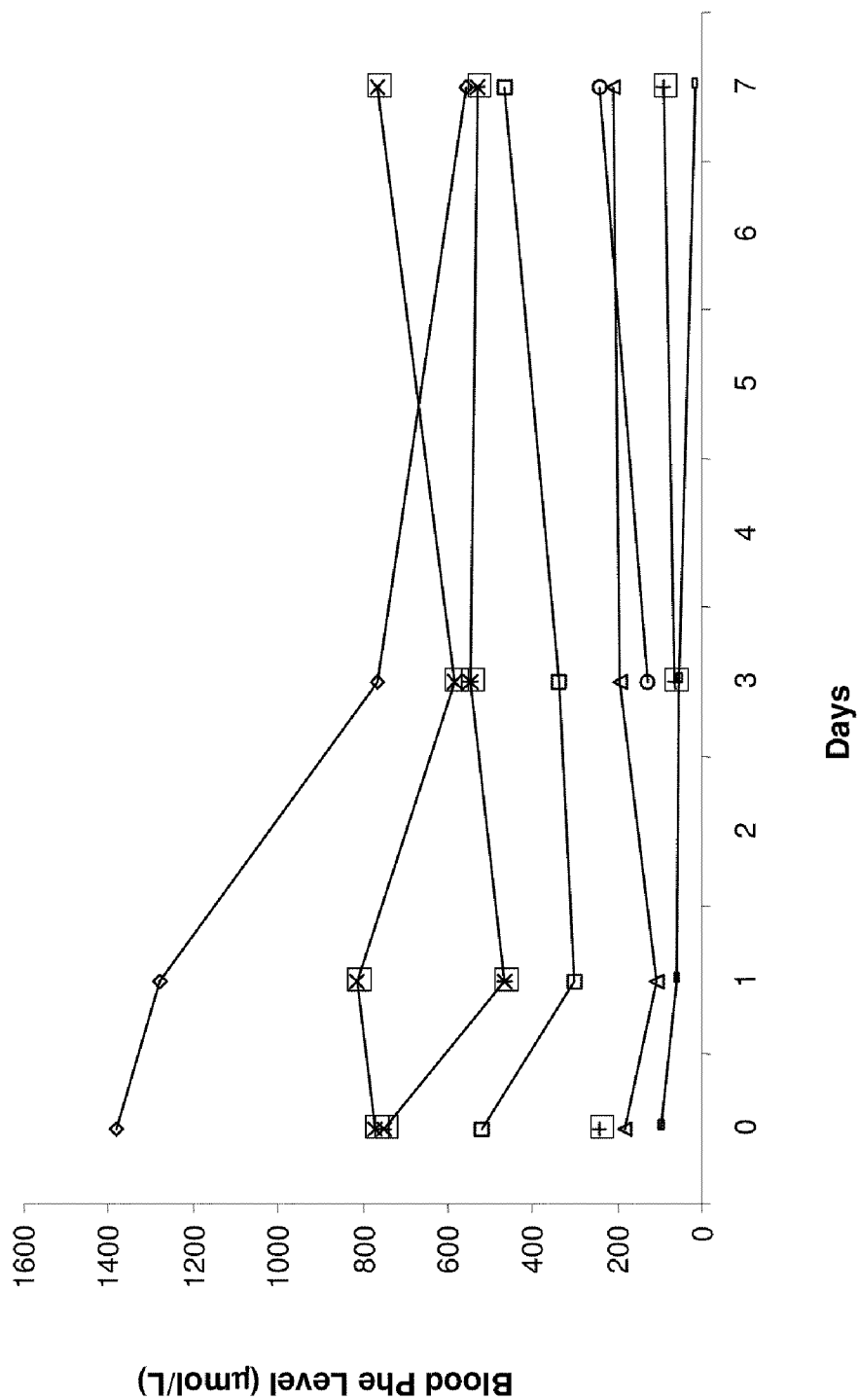


Figure 19

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# Individual Blood Phe in 8 Children with PKU on 20 mg/kg BH4 Daily

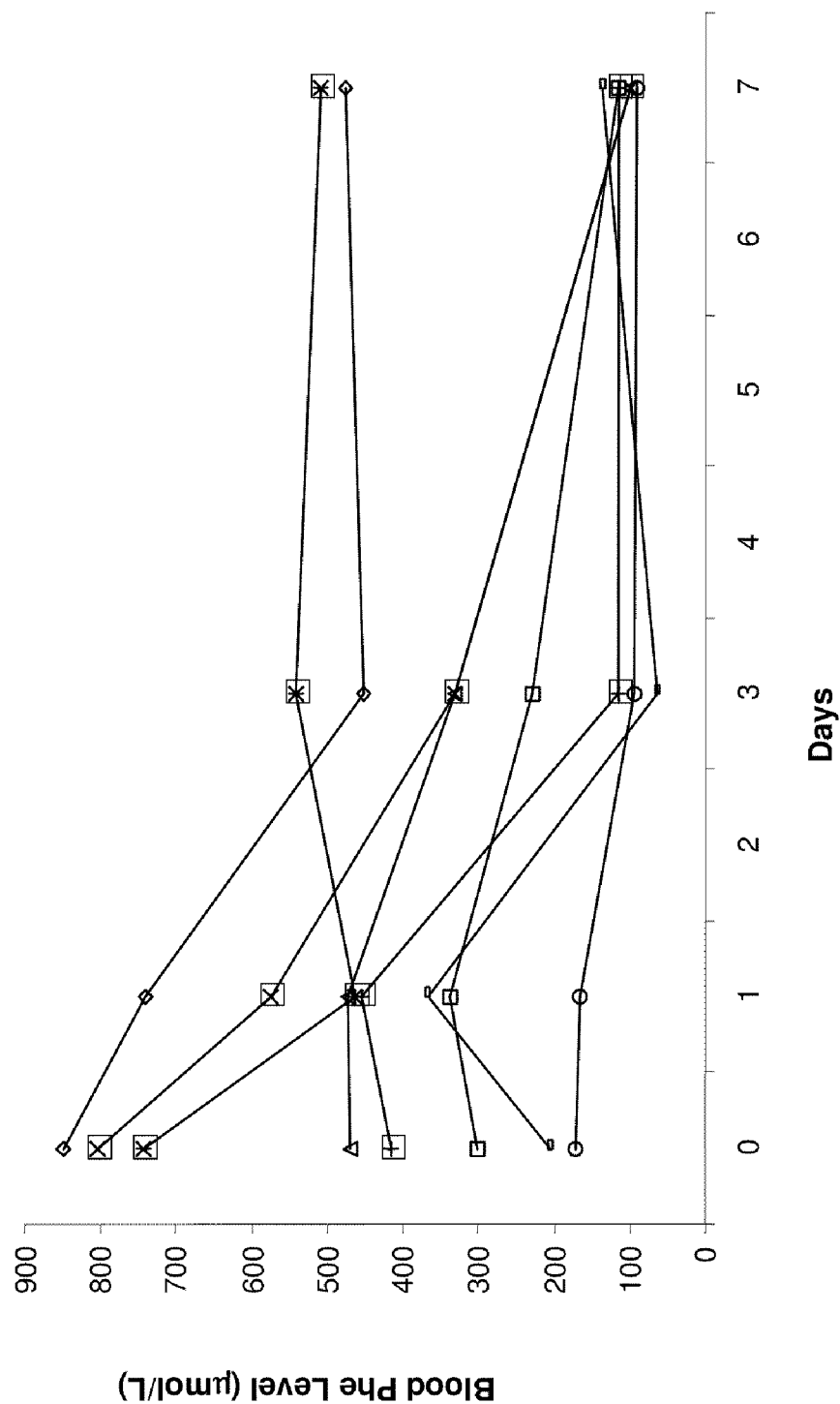


Figure 20

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## METHODS AND COMPOSITIONS FOR THE TREATMENT OF METABOLIC DISORDERS

### CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation of U.S. patent application Ser. No. 11/143,887, now U.S. Pat. No. 7,566,714 filed Jun. 1, 2005 which in turn is a continuation of U.S. patent application Ser. No. 10/991,573, which was filed on Nov. 17, 2004 now abandoned and which claimed the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application Ser. No. 60/520,767, which was filed Nov. 17, 2003. The entire disclosure of each of these priority applications is hereby incorporated herein by reference.

### BACKGROUND

#### 1. Field

The present invention is generally directed to the therapeutic intervention of metabolic disorders, particularly those involving amino acid metabolism. More particularly, the present invention is directed to methods and compositions for the treatment of phenylketonuria, vascular diseases, ischemic or inflammatory diseases, or insulin resistance, or conditions and patients that would benefit from enhancement of nitric oxide synthase activity.

#### 2. Background of the Related Technology

Phenylketonuria (PKU) is an inherited metabolic disorder that was first identified in the 1930s. In most cases, and until the mid-1990s, it was thought that this is a disorder of amino acid metabolism resulting from a deficiency in the liver enzyme phenylalanine hydroxylase (PAH). Deficiencies in PAH in turn result in an excess of phenylalanine (Phe) in the brain and plasma. The deficiency in PAH ultimately manifests in a lack of tyrosine, which is a precursor for the neurotransmitters.

Left undetected and untreated early in the life of an infant, PKU leads to irreversible damage of the nervous system, severe mental retardation and poor brain development. Features other than mental retardation in untreated patients include brain calcification, light pigmentation, peculiarities of gait, stance, and sitting posture, eczema, and epilepsy. It has been reported that an infant suffers a loss of 50 IQ points within the first year of infancy and PKU is invariably accompanied by at least some loss of IQ. Once detected, the condition is treated by providing the infant, and later the child, with a low Phe diet. In adults, the protein supplements routinely taken by classic PKU patients may be Phe-free with the assumption that such adults will receive sufficient quantities of Phe through the remaining diet, controlled under a strict regimen, so that the overall diet is a low Phe diet. Also, pregnant women who suffer from the condition are recommended a diet that is low in Phe to avoid the risk of impairment of the development of the fetus and congenital malformation (maternal PKU syndrome).

In more recent years it has been shown that pathological symptoms which manifest from the condition of excess of Phe, collectively termed hyperphenylalaninemia (HPA), may be divided into multiple discrete disorders, which are diagnosed according to plasma Phe concentrations and responsiveness to a co-factor for PAH. At an initial level, HPAs may be divided into HPA caused as a result of a deficiency in the cofactor 6R-L-erythro-5, 6, 7, 8, tetrahydrobiopterin (BH4; malignant PKU) and HPA resulting from a deficiency in PAH. The latter category is further subdivided into at least three categories depending on the plasma concentration of Phe in

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the absence of dietary or other therapeutic intervention (referred to herein as "unrestricted plasma Phe concentration").

Normal plasma Phe homeostasis is tightly controlled resulting in a plasma Phe concentration of  $60 \mu\text{mol/L} \pm 15 \mu\text{mol/L}$ . Classical PKU (OMIM No. 261600) is the most severe form of PKU and it results from null or severe mutations in PAH, which lead to unrestricted plasma Phe concentrations greater than  $1200 \mu\text{mol/L}$  when left untreated. Individuals with classical (or severe) PKU must be treated with a strict dietary regimen that is based on a very low Phe diet in order to reduce their Phe concentrations to a safe range. Milder forms of HPA also have been characterized. A less severe form of PKU is one which manifests in plasma Phe concentrations of  $10\text{--}20 \text{ mg/dL}$  ( $600\text{--}1200 \mu\text{mol/L}$ ), and is generally termed "mild PKU". This moderate form of PKU is managed through the use of moderate dietary restrictions, e.g., a low total protein diet, but otherwise not necessarily Phe-free. Finally, mild HPA, also referred to as benign or non-PKU HPA is characterized by plasma Phe concentrations of between  $180\text{--}600 \mu\text{mol/L}$ . The individuals with non-PKU HPA are not routinely treated as they are considered to have plasma Phe levels that are within the "safe" range. Nevertheless, as mentioned above, these Phe levels are still significantly elevated in these individuals as compared to normal, non-PKU subjects and may present detrimental sequelae in at least pregnant women and very young patients. For a more detailed review of HPA resulting from PAH deficiency, those of skill in the art are referred to Scriver et al., 2001 (*Hyperphenylalaninemia: Phenylalanine Hydroxylase Deficiency*, In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1667-1724). NIH Guidelines indicate that for children with PKU, it is preferable reduce the plasma Phe to be  $360\text{--}420 \mu\text{mol/L}$ .

HPA also results from defects in BH4 metabolism. BH4 is an essential cofactor of both tyrosine and tryptophan hydroxylase, the rate limiting enzymes in the biosynthesis of the neurotransmitters dopamine and serotonin. The effects of deficiencies in dopamine and serotonin are collectively known as "atypical" or "malignant" HPA. Thus, traditional diagnoses of HPA have involved a determination of whether the HPA is a result of BH4 deficiency or PAH deficiency. Typically, diagnosis of PKU is established on the basis of a persistently elevated blood Phe concentration. Following a positive screen for elevated blood Phe (plasma Phe  $>120 \mu\text{mol/L}$ ; Weglage et al., *J. Inherit. Metab. Dis.*, 25:321-322, 2002), a differential screen is performed in which it is determined whether the elevated Phe is a result of BH4 deficiency or PAH deficiency. The differential diagnosis involves determining whether the elevated Phe concentration is decreased as a result of BH4 administration (BH4 loading test). The BH4 loading test typically involves a one-time load of BH4 e.g.,  $5\text{--}20 \text{ mg/kg}$  being administered to the subject who is on a normal (i.e., unrestricted) diet and determining whether the subject experiences a decrease in Phe levels (see e.g., Ponzzone et al., *Eur. J. Pediatr.* 152:655-661, 1993; Weglage et al., *J. Inherit. Metab. Dis.*, 25:321-322, 2002.)

Typically, individuals that respond to a BH4 loading test by a decrease in plasma Phe levels are diagnosed as having a defect in BH4 homeostasis. However, there have been various reports of patients with a BH4 responsive type of PAH deficiency (Kure et al., *J. Pediatr.* 135:375-378, 1999; Lasker et al., *J. Inherit. Metab. Dis.* 25:65-70, 2002; Linder et al., *Mol. Genet. Metab.* 73:104-106, 2001; Spaapen et al., *Mol. Genet. and Metabolism*, 78:93-99, 2003; Trefz et al., 2001). These subjects have plasma Phe levels that are typical of



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moderate PKU, i.e., less than 1000  $\mu\text{mol/L}$  and typically less than 600  $\mu\text{mol/L}$ . Patients that have severe classical PKU are not responsive to typical 24 hour BH4 loading tests (Ponzone et al., *N. Engl. J. Med* 348(17):1722-1723, 2003).

It has been suggested that individuals that are responsive to BH4 do not require dietary intervention, but rather should be treated with BH4. Likewise, the converse has been suggested for subjects that have been diagnosed as non-responsive to the BH4 loading test, i.e., these subjects should be treated with dietary restriction and not BH4 therapy. Ponzone et al. particularly noted that individuals that have severe phenylketonuria will not respond to BH4 therapy and such therapy should not be used on these patients (Ponzone et al., *N. Engl. J. Med* 348(17):1722-1723, 2003). Thus, presently there are divergent therapeutic regimens for treatment of HPA depending on whether or not the individual is responsive to BH4. Moreover, it has been suggested that very few patients will benefit from BH4 therapy. In fact, it is thought that the only individuals with a PAH-deficient form of HPA that will benefit from BH4 therapy are those with mild PKU. As these individuals will typically have Phe levels in the safe range (i.e., less than 600  $\mu\text{M}$ ), the disease state can be controlled using moderate dietary restriction (see Hanley, *N. Engl. J. Med* 348(17):1723, 2003). Thus, BH4 therapy either alone, or in combination with any other therapeutic intervention, has not been considered as a viable therapeutic intervention for the vast majority of individuals with HPA.

BH4 is a biogenic amine of the naturally-occurring pterin family. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. This is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (*Disorders of tetrahydrobiopterin and related biogenic amines*. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. 8th ed. New York: McGraw-Hill, 2001: 1275-1776). Despite the elucidation of the role of BH4 deficiency in HPA, treatment with BH4 has not been suggested because such treatment is very expensive, as high as \$30,000 per year for an adolescent or adult, as compared with \$6,000 for phenylalanine-restricted dietary therapy (Hanley, *N. Engl. J. Med* 348(17): 1723, 2003). Another significant problem with BH4 is that this compound is unstable and readily undergoes aerobic oxidation at room temperature (Davis et al., *Eur. J. Biochem.*, Vol 173, 345-351, 1988; U.S. Pat. No. 4,701,455) and has a shelf-life of less 8 hours at room temperature (Bernegger and Blau, *Mol. Genet. Metabol.* 77:304-313, 2002).

Thus, to date, dietary intervention is the typical therapeutic intervention used for all patients with severe classical PKU and in many patients with moderate PKU. Such dietary intervention typically entails restricting the patient to foodstuff that is composed of natural foods which are free from, or low in, Phe. However, in addition to eliminating Phe, such a dietary regimen eliminates many sources of other essential amino acids, vitamins and minerals. Consequently, without supplementation, such a diet provides inadequate protein, energy, vitamins and minerals to support normal growth and development. As PKU is a manifestation of a lack of tyrosine, which arises due to the lack of hydroxylation of phenylalanine, tyrosine becomes an essential amino acid and dietary supplements for PKU must contain a tyrosine supplement. Therefore, it is common to use nutritional formulas to supple-

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ment the diets of PKU patients. Also, for babies, it is common to use infant formulas which have a low Phe content as the sole or primary food source.

However, dietary protein restriction is at best an ineffective way of controlling PKU in many classes of patients. For example, treatment is of paramount importance during pregnancy because high Phe levels may result in intrauterine retardation of brain development. However, a low protein diet during pregnancy may result in retarded renal development and is thought to produce a subsequent reduction in the number of nephrons and potentially leads to hypertension in adulthood. (D'Agostino, *N. Engl. J. Med.* 348(17):1723-1724, 2003).

Poor patient compliance with a protein-restricted diet also is a problem. The Phe-free protein formulae available are bitter tasting making it difficult to ensure that the patient consumes sufficient quantities of the protein to maintain the required daily intakes of protein, amino acids, vitamins, minerals, and the like. This is particularly a problem with older children who may require up to 70 g, dry weight, of the formulas per day. For example, Schuett, V. E.; 1990; DHHS Publication No HRS-MCH-89-5, reports that more than 40% of PKU patients in the US of eight years or older no longer adhere to the dietary treatment. (U.S. Pat. No. 6,506,422). Many adolescent patients fail to rigorously follow the protein-restricted diet due to fears of peer attitude.

Thus, there remains a need for a therapeutic medicament to replace or supplement and alleviate the dietary restrictions under which a PKU patient is placed. The present invention is directed to addressing such a need.

#### SUMMARY OF THE INVENTION

The invention describes intervention in metabolic disorders, particularly those involving amino acid metabolism. More particularly, the present invention is directed to methods and compositions for the treatment of subjects exhibiting elevated phenylalanine levels, for example, subjects suffering from hyperphenylalanemia, mild phenylketonuria or classic severe phenylketonuria; and methods and compositions for the treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity; and methods and compositions for treatment of subjects suffering from vascular diseases, ischemic or inflammatory diseases, diabetes, or insulin resistance.

In one aspect, the invention describes methods of treating classic severe phenylketonuria (PKU) in a subject comprising administering to the subject a protein-restricted diet in combination with a composition comprising tetrahydrobiopterin (BH4) or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of the combined administration. In specific embodiments, the subject is one who does not manifest a deficiency in BH4 homeostasis. The subject may be an individual that does not manifest symptoms of L-dopa neurotransmitter deficiency.

A subject selected from treatment according to the methods of the invention will have an elevated plasma Phe concentration, such a concentration may be greater than 1800  $\mu\text{M/L}$  in the absence of the therapeutic. Other embodiments contemplate that has a plasma phenylalanine concentration of greater than 1000  $\mu\text{M}$  in the absence of a therapeutic regimen. In preferred embodiments, the combined administration methods of the invention decrease the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ . More preferred

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erably, it is decreased to less than 500  $\mu$ M. Even more preferably, the combined administration decreases the plasma phenylalanine concentration of the subject to  $360 \mu\text{M} \pm 15 \mu\text{M}$ .

The BH4 is preferably administered in an amount of between about 1 mg/kg to about 30 mg/kg, more preferably between about 5 mg/kg to about 30 mg/kg. The BH4 may be administered in a single daily dose or in multiple doses on a daily basis. In some embodiments, the BH4 therapy is not continuous, but rather BH4 is administered on a daily basis until the plasma phenylalanine concentration of the subject is decreased to less than 360  $\mu$ M. Preferably, wherein the plasma phenylalanine concentration of the subject is monitored on a daily basis and the BH4 is administered when a 10% increase in plasma phenylalanine concentration is observed. Preferably, the BH4 being administered is a stabilized crystallized form of BH4 that has greater stability than non-crystallized stabilized BH4. More preferably, the stabilized crystallized form of BH4 comprises at least 99.5% pure 6R BH4. Precursors such as dihydrobiopterin (BH2), and sepiapterin also may be administered. BH4 may be administered orally

The protein-restricted diet administered in the methods herein is one that is a phenylalanine-restricted diet wherein the total phenylalanine intake of the subject is restricted to less than 600 mg per day. In other embodiments, the protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 300 mg per day. In still other embodiments, the protein-restricted diet is one which is supplemented with amino acids, such as tyrosine, valine, isoleucine and leucine. In certain embodiments, protein-restricted diet comprises a protein supplement and the BH4 is provided in the same composition as the protein supplement.

In specific embodiments, the subject is one which has been diagnosed as having a mutant phenylalanine hydroxylase (PAH). The mutant PAH may comprise a mutation in the catalytic domain of PAH. Exemplary such mutations include one or more mutations selected from the group consisting of F39L, L48S, 165T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C.

Also contemplated herein is a method for the treating a pregnant female having hyperphenylalaninemia (HPA) comprising administering to the subject a protein-restricted diet in combination with a composition comprising tetrahydrobiopterin (BH4) or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the subject as compared to the concentration in the absence of the combined administration. In certain embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 180  $\mu$ M but less than 600  $\mu$ M. In other embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 500  $\mu$ M but less than 1200  $\mu$ M. In still other embodiments, the subject has an unrestricted plasma phenylalanine concentration of greater than 1000  $\mu$ M.

Also contemplated is a method of treating a patient having above normal concentration of plasma phenylalanine (e.g., greater than 180  $\mu$ M/L and more preferably, greater than 360  $\mu$ M/L) comprising administering to the patient a stabilized BH4 composition in an amount effective to produce a decrease in the plasma phenylalanine concentration of the patient. Preferably, the stabilized BH4 composition is stable at room temperature for more than 8 hours. The patient will likely have a plasma phenylalanine concentration greater than 180  $\mu$ M prior to administration of the BH4. More particularly,

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the patient has a plasma phenylalanine concentration of between 120  $\mu$ M and 200  $\mu$ M. In other embodiments, the patient has a plasma phenylalanine concentration of between 200  $\mu$ M and 600  $\mu$ M. In still other embodiments, the patient has a plasma phenylalanine concentration of between 600  $\mu$ M and 1200  $\mu$ M. Yet another class of patients to be treated are those that have an unrestricted plasma phenylalanine concentration greater than 1200  $\mu$ M. In specific embodiments, the patient is an infant, more particularly, an infant having a plasma phenylalanine concentration greater than 1200  $\mu$ M. In other embodiments, the patient is pregnant and pregnant patient has a plasma phenylalanine concentration of between about 200  $\mu$ M to about 600  $\mu$ M. Pregnant patients with a plasma phenylalanine concentration greater than 1200  $\mu$ M are particularly attractive candidates for this type of therapy, as are patient who are females of child-bearing age that are contemplating pregnancy. In those embodiments, in which the patient has a plasma phenylalanine concentration greater than 1200  $\mu$ M, and the method further comprises administering a protein-restricted diet to the patient.

The invention also contemplates a method of treating a patient having phenylketonuria, comprising administering to the patient a stabilized BH4 composition in an amount effective to produce a decrease in the plasma phenylalanine concentration of the patient wherein the patient has been diagnosed as unresponsive to a single-dose BH4 loading test. Preferably, the patient is unresponsive within 24 hours of the BH4 load.

Another related aspect of the invention provides a multiple dose loading test that involves administration of more than one dose of BH4. The data described herein demonstrates that subjects who are considered "unresponsive" to a single dose BH4 loading test can respond to multiple doses of BH4 with a significant reduction in phenylalanine levels. In one embodiment, at least two doses of BH4 which may be between about 5 mg to 40 mg are administered to a subject over a time period of more than one day, preferably 7 days.

The treatment methods according to the invention may comprise administering between about 10 mg BH4/kg body weight to about 200 mg BH4/kg body weight. The BH4 may be administered through any route commonly used in practice, e.g., orally, subcutaneously, sublingually, parenterally, per rectum, per and nares. The BH4 may be administered daily or at some other interval, e.g., every alternative day or even weekly. The BH4 is preferably administered in combination with a protein-restricted diet, and optionally concurrently with folates, including folate precursors, folic acids, and folate derivatives.

It is contemplated that that BH4 will be administered as part of a component of a therapeutic protein formulation. The protein-restricted diet may comprise a normal diet of low-protein containing foodstuff. Alternatively, the protein-restricted diet comprises an intake of protein formula that is phenylalanine-free protein diet, and the subject obtains his essential amount of Phe from remaining components of a very low protein diet. In certain embodiments, the protein-restricted diet is supplemented with non-phenylalanine containing protein supplements. More particularly, the non-phenylalanine containing protein supplements comprise tyrosine or other essential amino acids. In other embodiments, the protein supplements may also comprise folates, including folate precursors, folic acids, and folate derivatives.

The invention contemplates methods of treating an infant having phenylketonuria, comprising administering a stabilized BH4 composition to the patient in an amount effective to produce a decrease in the plasma phenylalanine concentration of the infant wherein the infant is between 0 and 3 years

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of age and the infant has a plasma phenylalanine concentration of between about 360  $\mu$ M to about 4800  $\mu$ M. Prior to the administering of BH4, the infant has a phenylalanine concentration of about 1200  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 1000  $\mu$ M. In other embodiments, prior to the administering of BH4 the infant has a phenylalanine concentration of about 800  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 600  $\mu$ M. In still further embodiments, prior to the administering of BH4 the infant has a phenylalanine concentration of about 400  $\mu$ M and the administering of BH4 decreases the plasma phenylalanine concentration to about 300  $\mu$ M. The therapeutic methods contemplated herein should preferably reduce the plasma phenylalanine concentration of the infant to  $360 \pm 15$   $\mu$ M.

Also contemplated is a composition comprising a stabilized, crystallize form of BH4 that is stable at room temperature for more than 8 hours and a pharmaceutically acceptable carrier, diluent or excipient. The composition may further comprise a medical protein supplement. In other embodiments, the BH4 composition is part of an infant formula. In still other embodiments, the protein supplement is phenylalanine free. The protein supplement preferably is fortified with L-tyrosine, L-glutamine, L-carnitine at a concentration of 20 mg/100 g supplement, L-taurine at a concentration of 40 mg/100 g supplement and selenium. It may further comprise the recommended daily doses of minerals, e.g., calcium, phosphorus and magnesium. The supplement further may comprise the recommended daily dose of one or more amino acids selected from the group consisting of L-leucine, L-proline, L-lysine acetate, L-valine, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-tryptophan, L-serine, L-threonine, L-histidine, L-methionine, L-glutamic acid, and L-aspartic acid. In addition, the supplement may be fortified with the recommended daily dosage of vitamins A, D and E. The supplement preferably comprises a fat content that provides at least 40% of the energy of the supplement. Such a supplement may be provided in the form of a powder supplement or in the form of a protein bar.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further illustrate aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of the specific embodiments presented herein.

FIG. 1 is a powder X-ray diffraction pattern of (6R)-BH4 Form B.

FIG. 2 is a powder X-ray diffraction pattern of (6R)-BH4 Form A.

FIG. 3 is a powder X-ray diffraction pattern of (6R)-BH4 Form F.

FIG. 4 is a powder X-ray diffraction pattern of (6R)-BH4 Form J.

FIG. 5 is a powder X-ray diffraction pattern of (6R)-BH4 Form K.

FIG. 6 is a powder X-ray diffraction pattern of (6R)-BH4 Form C.

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FIG. 7 is a powder X-ray diffraction pattern of (6R)-BH4 Form D.

FIG. 8 is a powder X-ray diffraction pattern of (6R)-BH4 Form E.

FIG. 9 is a powder X-ray diffraction pattern of (6R)-BH4 Form H.

FIG. 10 is a powder X-ray diffraction pattern of (6R)-BH4 Form O.

FIG. 11 is a powder X-ray diffraction pattern of (6R)-BH4 Form G.

FIG. 12 is a powder X-ray diffraction pattern of (6R)-BH4 Form I.

FIG. 13 is a powder X-ray diffraction pattern of (6R)-BH4 Form L.

FIG. 14 is a powder X-ray diffraction pattern of (6R)-BH4 Form M.

FIG. 15 is a powder X-ray diffraction pattern of (6R)-BH4 Form N.

FIG. 16 is a mean blood phenylalanine level comparison at time zero, 3 days, and 7 days for multiple daily BH4 doses of 10 mg/kg/d and 20 mg/kg/d.

FIG. 17 is a comparison of daily individual blood phenylalanine levels for 12 adults having PKU and taking 10 mg/kg/d over 7 days.

FIG. 18 is a comparison of daily individual blood phenylalanine levels for 12 adults having PKU and taking 20 mg/kg/d over 7 days.

FIG. 19 is a comparison of daily individual blood phenylalanine levels for 8 children having PKU and taking 10 mg/kg/d over 7 days.

FIG. 20 is a comparison of daily individual blood phenylalanine levels for 8 children having PKU and taking 20 mg/kg/d over 7 days.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

Dietary intervention is the therapeutic intervention used for all patients with severe classical PKU and in many patients with moderate PKU. However, such dietary protein restriction leads to an inadequate supply of protein, energy, vitamins and minerals to support normal growth and development. Thus, dietary protein restriction is at best an ineffective way of controlling the PKU in many classes of patients, especially in pregnant women and in young children, both categories of subjects that require elevated amounts of protein as compared to normal adult individuals. Use of dietary restriction also is hampered by poor patient compliance with a protein-restricted diet. In October 2000, the National Institutes of Health issued a consensus statement on PKU screening and management in which "research on nondietary alternatives to treatment of PKU [was] strongly encouraged." Thus, there is an art-recognized need for a therapeutic medicament to replace and/or supplement and alleviate the dietary restrictions under which a PKU patient is placed.

The present application for the first time describes a pharmaceutical intervention of PKU based on the administration of a stabilized form of BH4. The methods and compositions for producing such a stabilized BH4 compositions are described in further detail in Example 2. The stabilized BH4 compositions of the present invention comprise BH4 crystals that are stable at room temperature for longer than 8 hours. The methods and compositions of the present invention contemplate pharmaceutical compositions of the stabilized BH4 alone that may be delivered through any conventional route of administration, including but not limited to oral, intramuscular injection, subcutaneous injection, intravenous injection



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and the like. The compositions of the present invention may further comprise BH4 compositions in combination with an antioxidant that aids in prolonging the stability of the BH4 composition. In addition, discussed in greater below, the present invention further comprises foodstuffs that comprise BH4. For example, the invention contemplates conventional protein powder compositions such as PHENEX, LOFENALAC, PHENYL-FREE and the like that have been modified by the addition of BH4.

The present invention further contemplates the therapeutic intervention of various PKU phenotypes by administration of BH4 in combination with a protein-restricted diet. The BH4 to be administered in combination with the diet may, but need not necessarily, be a stabilized BH4 composition described herein. Those of skill in the art are aware of methods of producing a BH4 composition that is unstable at room temperature and in light. While therapies using such a composition are hindered by the instability of the BH4 composition, its use is still contemplated in certain combination therapies where BH4 non-responsive patients suffering from severe classical PKU are treated with a course of BH4 treatment and dietary protein restriction.

Methods and compositions for effecting the treatment of metabolic disorders, including PKU, are described in further detail herein below.

#### I. PATIENTS TO BE TREATED

The present invention is directed to the treatment of a variety of HPA patient populations with methods that comprise the use of stabilized BH4 compositions, or unstabilized BH4 compositions, either alone or in combination with other therapeutic regimens, for managing HPA and/or PKU. In particular, it is contemplated that any type of BH4, in a stabilized or other form may be used to treat that patient population that has phenylalanine concentrations that are low enough that dietary intervention is not normally used (i.e., patients with mild HPA). Such patients that are amenable to all forms treatment with BH4 compositions to ameliorate the effects of mild HPA, include pregnant women and infants with serum concentrations of less than 200  $\mu$ M. The various patient populations, and their different therapeutic needs, are discussed in further detail in the present section.

Certain embodiments of the present invention are directed to treating classic severe PKU by administering to the subject a protein-restricted diet in combination with a composition comprising BH4 or a precursor or derivative thereof, wherein the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of said subject as compared to said concentration in the absence of said combined administration. In addition, the invention also contemplates treating a pregnant female that has HPA by administering to the female a protein-restricted diet in combination with BH4 or a precursor or derivative thereof, such that the combined administration of the protein-restricted diet and BH4 is effective to lower the phenylalanine concentration in the plasma of the pregnant woman as compared to such a concentration in the absence of said combined administration. In specific embodiments, therapy is contemplated for patient who manifest Phe levels greater than 420  $\mu$ M

Other embodiments of the invention entail administering a stabilized BH4 composition to any individual that has HPA, characterized by a plasma Phe concentration greater than 180  $\mu$ M prior to the administration of the BH4, in an amount effective to produce a decrease in such a plasma Phe concentration of the patient. The methods of the invention also may

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be used in the treatment of PKU patients that have been diagnosed as unresponsive to a BH4 loading test. The methods of the invention will be useful in treating an infant having PKU characterized by an elevated Phe concentrations of between greater than 300  $\mu$ M/L with the stabilized BH4 compositions described herein. By "infant" the present application refers to a patient that is between the ages of 0 to about 36 months.

The data described herein demonstrates that subjects who are considered "unresponsive" to the single dose BH4 loading test may in fact respond to multiple doses of BH4 with a significant reduction in phenylalanine levels. Thus, another aspect of the invention provides a multiple dose loading test that involves administration of more than one dose of BH4. Exemplary multiple dose loading tests include administration of between 5 and 40 mg/kg tetrahydrobiopterin, or more preferably 10 to 20 mg/kg, over a time period of at least 1 day, or at least 2 days, or at least 3, 4, 5, 6, 7, 10 or 14 days, preferably 2-14, 3-14, or 5-10 days, and most preferably 7 days.

The invention provides methods of using any of the tetrahydrobiopterin polymorphs described herein, or stable pharmaceutical preparations comprising any of such polymorphs, for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described elsewhere herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal.

The invention further provides methods of using any of the polymorphs described herein, or stable pharmaceutical preparations comprising any of such polymorphs, for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from treatment with folates, including folate precursors, folic acids, or folate derivatives. Thus, it is also contemplated, that compositions and methods disclosed herein include the concurrent treatment with folates, including folate precursors, folic acids, or folate derivatives. Exemplary folates are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folypolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folypolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-

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tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and salts thereof. as is treatment with a pharmaceutical composition or foodstuff that comprises both a tetrahydrobiopterin polymorph and a folate.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (narrowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, such as hypertension, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, peripheral circulation disorder, coronary arteriovenous anastomosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arteriosclerotic heart diseases or the like so far as they are associated with insulin resistance. It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

#### A. Characteristics of Severe Classical PKU and Methods of Treatment Thereof According to the Present Invention

As indicated herein above in the background section, severe PKU manifests in a plasma Phe concentration greater than 1200  $\mu\text{M/L}$  and may be found to be as high as 4800  $\mu\text{M/L}$ . Patients that have this disorder must be treated with a Phe-free diet in order to bring their plasma Phe concentrations down to a level that is clinically acceptable (typically, less than 600  $\mu\text{M/L}$ , and preferably less than 300  $\mu\text{M/L}$ ). These patients are only able to tolerate a maximum of between 250-350 mg dietary Phe per day (Spaapen et al., *Mol. Genet and Metab.* 78:93-99, 2003). As such, these patients are started on a Phe-restricted formula diet between 7-10 days after birth and are burdened with this dietary restriction for the remainder their lifespan. Any alleviation of the strict dietary restrictions that these individuals are encumbered with would be beneficial.

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The tests used for the diagnosis of individuals with classical Phe are described in further detail below in Section III. These tests have revealed that patients with classical severe PKU are non-responsive to BH4 and require a low phenylalanine diet (Lucke et al., *Pediatr. Neurol.* 28:228-230, 2003). In the present invention however, it is contemplated that this class of PKU patients should be treated with BH4 in order that the need for a strict phenylalanine-free diet may be alleviated.

Thus, it is contemplated that the methods of the invention will entail determining that the patient is suffering from classical PKU by monitoring the plasma Phe concentration of the individual. The patient is then treated by administering a combined regimen of a low protein diet and BH4 such that there is produced at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe concentration. Even more preferably, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with severe classical PKU has a Phe concentration of 4800  $\mu\text{M/L}$ , a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 480  $\mu\text{M/L}$ , a concentration that is sufficiently low to require little dietary restriction). Of course, it should be understood that the treatment methods of the present invention (whether for treating severe classical PKU or any other HPA described herein), should attempt to lower the plasma Phe concentrations of the patient to levels as close to 360  $\mu\text{M/L} \pm 15 \mu\text{M/L}$  as possible.

In preferred embodiments the plasma Phe concentrations of the classical PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is greater than 1000  $\mu\text{M/L}$  to any plasma Phe level that is less than 600  $\mu\text{M/L}$ . Of course, even if the combined treatment with the BH4 and the protein-restricted diet produces a lesser decrease in plasma Phe concentration, e.g., to a level of between 800  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$ , this will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-restricted formula, thereby resulting in a marked improvement in the quality of life of the individual, as well as leading to greater patient compliance with the dietary restriction.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy, the patient will be able to increase his/her intake of dietary Phe from 250-350 mg/day to 350-400 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a classic PKU patient to a moderate PKU patient). Of course, it would be preferable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 250-350 mg/day to 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a classic PKU patient to a mild PKU patient), or even more preferably, to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

#### B. Characteristics of BH4-Non Responsive PKU Patients and Methods of Treatment Thereof According to the Present Invention

A second group of patients that can be treated with the methods of the present invention are those individuals that have been determined to have an elevated plasma Phe concentrations i.e., any concentration that is greater than 200  $\mu\text{M/L}$ , but have been diagnosed to be non-responsive to BH4

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therapy (as determined by the BH4 loading test described below). Such patients may include those individuals that have mild PKU (i.e., plasma Phe concentrations of up to phenylalanine concentrations are determined by electrospray ionization mass spectrometry before Phe loading as well as before, and at 4, 8, and 15 hours after BH4 loading. Newborns may be breast fed, whereas older patients are give a standardized protein intake (10 mg Phe/kg) between 6-8 hours after BH4 loading. Muntau also describe methods for Phe oxidation. After a 4-hour fast and an overnight fast a total of 6 mg/kg 13C labeled Phe dissolved in dextrose solution is administered orally. Breath samples are then collected over a period of 180 minutes and stored in evacuated glass tubes. The samples are then analysed using isotope ration mass spectrometry and the recovery of carbon 13 is calculated (Treacy et al., *Pediat. Res.* 42:430-5, 1997)

Muntau et al. classify patients as BH4 responsive when the blood Phe levels 15 hours post-BH4 challeng have decreased by more than 30% from the value obtained prior to the BH4 administration. An improvement in the rate of Phe oxidation, as determined by measurements of carbon dioxide obtained during the 180 minutes of testing, was considered significant when the supplementation with BH4 increased the value of Phe oxidation by at least 15%.

Those of skill in the art will be able to use any of the above-referenced methods to determine whether an individual will be responsiveness to BH4. However, other equivalent and related methods for determining BH4 responsiveness also may be known to those of skill in the art and may be used instead of the methods described above.

#### B. Determination of Phe Concentrations

There are numerous methods for determining the presence of Phe in blood (Shaw et al., *Analytical Methods in Phenylketonuria-Clinical Biochemistry*, In Bickett et al. Eds. *Phenylketonuria and Some Other Inborn Errors of Amino Acid Metabolism*, Stuttgart, Georg Thiem Verlag, 47-561971.). Typically, phenylalanine and tyrosine concentrations are determined from the serum of a patient using a fluorometric assay. This assay relies on the formation of fluorescent substance when phenylalanine is heated with ninhydrin in the presence of leucylalanine (McCaman et al., *J. Lab. Clin. Med.* 59:885-890, 1962.) 600  $\mu$ M/L), individuals that have moderate PKU (i.e., plasma Phe concentrations of between 600  $\mu$ M/L to about 1200  $\mu$ M/L), as well as patients that have classic severe PKU (i.e., plasma Phe concentrations that are greater than 1200  $\mu$ M/L).

The patients that are non-responsive to BH4 therapy are given BH4 in combination with a reduced amount of protein in their diet in order to decrease the plasma Phe concentrations of the patient. The methods of the present invention are such that the administration of the BH4 therapy produces a greater decrease in the plasma Phe concentrations of the patient as compared to the decrease that is produced with the same dietary protocol administered in the absence of the BH4 therapy.

In preferred embodiments, the patients are administered a composition that comprises a stabilized, crystallized form of BH4 characterized in Example 2 described herein below. This BH4 composition differs from those previously available in the art in that it is more stable at room temperature than the preparations previously known to those of skill in the art, e.g., those available in the BH4 loading kits (Schircks Laboratories, Jona, Switzerland.) Thus, the BH4 formulation may be stored at either room temperature or refrigerated and retain greater potency than the previously available BH4 compositions. As such, it is contemplated that this form of BH4 will have a greater therapeutic efficacy than a similar concentra-

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tion the previously available BH4 compositions. This greater efficacy may be used to produce a therapeutically effective outcome even in patients that were previously identified as being non-responsive to BH4.

As with the subset of patients described in Section IA above, the BH4 non-responsive patients described in the present section may be treated by the stabilized BH4 compositions either alone or in combination with dietary restrictions. The dietary restrictions may be as a diet that restricts the Phe intake by providing a synthetic medical protein formula that has a diminished amount of Phe or alternatively, the dietary restriction may be one which simply requires that the patient limit his/her overall protein intake but nevertheless allows the patient to eat normal foodstuffs in limited quantities.

The preferred therapeutic outcomes discussed for classical PKU patients in Section IA above are incorporated into the present section by reference. Preferred therapeutic outcomes for patients with moderate PKU (i.e., patients that has an unrestricted plasma Phe concentration of 600  $\mu$ M/L to 1200  $\mu$ M/L) include at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe concentration. Even more preferably, the method will produce a 40%, 50%, 60%, 70%, 80%, 90% or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with moderate classical PKU has a Phe concentration of 1000  $\mu$ M/L, a 90% decrease in the Phe concentration will produce a plasma Phe concentration of 100  $\mu$ M/L, a concentration that is sufficiently low to require little dietary restriction).

In preferred embodiments, the plasma Phe concentrations of the moderate PKU patient being treated is reduced from any amount of unrestricted plasma Phe concentration that is between 600  $\mu$ M/L to 1200  $\mu$ M/L to any plasma Phe level that is less than 300  $\mu$ M/L. A particularly preferred treatment with the BH4 (either alone or in combination with a dietary restriction) produces a decrease in plasma Phe concentration, e.g., to a level of between 200  $\mu$ M/L to about 400  $\mu$ M/L, which will be viewed as a clinically useful outcome of the therapy because patients that have a plasma Phe concentration in this range can manage the disease by simply restricting the amount of protein in the diet as opposed to eating a Phe-restricted formula. Indeed, in many studies, it is taught that such patients may even eat a normal diet.

Any increase in the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 350-400 mg/day to 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a moderate PKU patient to a mild PKU patient). Of course, it would be preferable that the therapeutic intervention taught herein would allow the patient to increase his/her intake of dietary Phe from 350-400 mg/day to 400 to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

Even if the patient being treated is one who manifests only mild PKU, i.e., has a dietary allowance of 400-600 mg Phe intake/day) will benefit from the BH4-based therapies of the present invention because it is desirable to produce a normalized plasma Phe concentration that is as close to 360  $\mu$ M/L  $\pm$  15  $\mu$ M/L as possible. For such patients, a preferred therapeutic outcomes will include at least a 25% decrease in the plasma Phe concentrations of the patient. Preferably, the method will produce a 30% decrease in the plasma Phe con-



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centration. Even more preferably, the method will produce a 40%, 50%, 60%, or greater decrease in the plasma Phe concentration of the individual (for example, where a patient with mild PKU has a Phe concentration of 600  $\mu\text{M/L}$ , a 60% decrease in the Phe concentration will produce a plasma Phe concentration of 360  $\mu\text{M/L}$ , i.e., an acceptable, normal concentration of plasma Phe).

In preferred embodiments, the plasma Phe concentrations of the mild PKU patient being treated is reduced from any amount of non-restricted plasma Phe concentration that is between 400  $\mu\text{M/L}$  to 600  $\mu\text{M/L}$  to any plasma Phe level that is less than 100  $\mu\text{M/L}$ . Of course, even if the treatment with the BH4 (either alone or in combination with a dietary restriction) produces a lesser decrease in plasma Phe concentration, e.g., to a level of between 200  $\mu\text{M/L}$  to about 400  $\mu\text{M/L}$ , this will be viewed as a clinically useful outcome of the therapy.

Any increase the amount of dietary Phe levels that can be tolerated by the patient as a result of the treatment will be considered to be a therapeutically effective outcome. For example, it is contemplated that as a result of administering the BH4-based therapy (either alone or in combination with other therapeutic intervention), the patient will be able to increase his/her intake of dietary Phe from 400-600 mg/day (i.e., the Phe tolerance phenotype of the patient is altered from that of a mild PKU patient to a mild HPA patient) to allow the patient to have an intake of greater than 600 mg Phe/day (i.e., normal dietary intake).

Furthermore, even if the patient is one who only manifests the symptoms of non PKU HPA, i.e., has an elevated plasma Phe concentration of up to 600  $\mu\text{M/L}$ , but is otherwise allowed to eat a normal protein diet will benefit from the BH4 therapies of the invention because it has been shown that elevated Phe concentrations have significant effects on the IQ of such individuals. Moreover, as discussed below, BH4-based therapeutic intervention of subjects with special needs, e.g., pregnant women and infants, is particularly important even if that patient's plasma Phe levels are within the perceived "safe" level of less than 200  $\mu\text{M/L}$ .

C. Maternal PKU and Methods of Treatment Thereof According to the Present Invention

Metabolic control of plasma Phe levels in PKU women planning conception and those who are pregnant is important because of the serious consequences to the fetus exposed to even moderately elevated Phe levels in utero, regardless of the PAH status of the fetus. Therapeutic control of plasma Phe concentration is especially important in the first trimester of pregnancy, as failure to achieve adequate control will result in disorders including microcephaly, mental deficiency and congenital heart disease.

For example, the NIH Consensus Statement (vol 17 #3, October 2000) on Phenylketonuria reported that exposure of a fetus to maternal Phe levels of 3-10 mg/dL produced a 24% incidence of microcephaly, whilst those exposed to greater than 20 mg/dL (i.e., greater than 1200  $\mu\text{M/L}$ ) had a 73% incidence of microcephaly. Likewise congenital heart disease was found in over 10% of children exposed to maternal Phe levels that were greater than 20 mg/dL. Importantly, it has been noted that levels of Phe greater than 6 mg/dL significantly decrease the IQ of the child. Thus, it is imperative to ensure that the plasma Phe concentration of women with all forms of phenylketonuria, even those manifesting the mildest HPA, must be tightly controlled in order to avoid the risk of maternal PKU syndrome. However, the acceptable target levels for the plasma Phe concentrations of PKU women that have been used in U.S. clinics have ranged between 10 mg/dL and 15 mg/dL, which are much higher than the 2-6 mg/dL levels recommended for pregnant women or the 1-4 mg/dL

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that are used in British and German clinics to diminish the risks of developing maternal PKU syndrome.

Another important consideration for pregnant women is their overall protein intake. During pregnancy, it is important that women eat sufficient protein because it has been suggested that a low protein diet during pregnancy will result in retarded renal development and subsequent reduction in the number of nephrons and potentially leads to hypertension in adulthood. (D'Agostino, *N. Engl. J. Med.* 348(17)1723-1724, 2003). The following table provides exemplary guidelines for the recommended total dietary protein intake for various individuals.

TABLE

United States Guidelines for dietary protein requirements		
	Age	Recommended Total Protein Intake (g)
Infant	6 months or less	13
	6 months-1 year	14
	1-3 years	16
Children	4-6 years	24
	7-10 years	28
Males	11-14 years	45
	15-18 years	59
	19-24	58
	25-50	63
	51+	63
Females	11-14 years	46
	15-18 years	44
	19-24	46
	25-50	50
	51+	50
Pregnant		60
Lactating		65

The actual amount of protein ingested depends on the Phe content of the protein. The amino acid profiles of plant proteins is different from animal proteins. For example, with a focus on starches and vegetables, a general rule of 45-50 mg/Phe per gram of protein may suffice. However, an accepted standard for evaluating the constituents amino acids of a protein is an egg white, which contains 3.5 grams of protein of which 204 mg is Phe.

As can be seen from the above exemplary guidelines, in the United States, the recommended protein intake for women of child-bearing age (e.g., less than 51) is from about 44 to 50 g/day, whereas pregnant women require are recommended an intake of about 60 g/day. In Canada and the United Kingdom, the recommended protein intake for pregnant women is in the order of about 70 g/day and 52 g/day. Thus, the need to ensure that the plasma Phe concentration levels of pregnant women are tightly controlled is further complicated by the fact that this group of PKU patient requires more protein than non-pregnant PKU females of comparable age.

In view of the above, it is contemplated that BH4-based therapies of the present invention will be particularly useful in pregnant women. It is contemplated that a woman suffering from any form of HPA who is pregnant or is contemplating pregnancy will be placed on a course of BH4 therapy to ensure that her plasma Phe concentration levels are maintained as close to 180  $\mu\text{M/L}$  to about 360  $\mu\text{M/L}$  as possible. Such a course of therapy will preferably allow that woman to increase her level of normal protein intake.

The discussion of levels of plasma Phe concentrations and the degrees to which such Phe concentrations should be decreased discussed herein above in Sections IA and IB are incorporated into the present section for pregnant women.

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#### D. Managing PKU in Infants and Methods of Treatment Thereof According to the Present Invention

As discussed herein throughout, it has been determined that an elevation in the plasma Phe concentration in infants (ages zero to 3 years old) results in significant drop in IQ of the child. However, as has been discussed elsewhere in the specification, patients that have an elevated plasma Phe concentration of anywhere up to 400  $\mu\text{M/L}$  do not normally receive any dietary intervention. Thus, infants at the age of zero to 3 years in age suffer from significant deleterious effects from the present therapies. The instant application contemplates treating any infant having an unrestricted plasma Phe concentration that is greater than 360  $\mu\text{M/L} \pm 15$   $\mu\text{M/L}$  with a therapeutic composition that comprises BH4 in order to produce a beneficial decrease the plasma Phe concentration of that subject.

In preferred embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of about 1200  $\mu\text{M/L}$  prior to the administration of BH4 and said administration decreases the plasma Phe concentration. Preferably, the plasma Phe concentration is decreased to from greater than 1800 to about 1500  $\mu\text{M/L}$ , about 1200  $\mu\text{M/L}$ , about 1100  $\mu\text{M/L}$ , about 1000  $\mu\text{M/L}$ , about 900  $\mu\text{M/L}$ , about 800  $\mu\text{M/L}$ , about 700  $\mu\text{M/L}$ , about 600  $\mu\text{M/L}$ , about 550  $\mu\text{M/L}$ , about 500  $\mu\text{M/L}$ , about 450  $\mu\text{M/L}$ , 400  $\mu\text{M/L}$ , about 350  $\mu\text{M/L}$ , about 300  $\mu\text{M/L}$ , about 275  $\mu\text{M/L}$ , about 250  $\mu\text{M/L}$  upon administration. In other embodiments, the infant is aged between zero and 3 years of age and has an unrestricted plasma Phe concentration of greater than 1200  $\mu\text{M/L}$  and preferably, this plasma Phe concentration is decreased to about 800  $\mu\text{M/L}$ , or more preferably to about 500  $\mu\text{M/L}$  or even more preferably to about 360  $\mu\text{M/L}$  upon administration of BH4, either alone or in combination with diet. Those of skill in the art would understand that the invention contemplates treating infants with unrestricted plasma Phe concentrations of greater than 360  $\mu\text{M/L}$  with BH4 to produce decreases in such plasma Phe concentrations. The discussion of therapeutic reductions of plasma Phe concentrations in Sections IA and IB above are incorporated herein by reference. Further, any decrease over 10% of the initial unrestricted plasma Phe concentration will be considered a therapeutic outcome for the therapeutic regimens for the infants. It should be understood that the BH4 therapies may be combined with dietary restrictions to effect the therapeutic decrease in plasma Phe concentrations in such infants.

## II. COMPOSITIONS FOR USE IN THE TREATMENT

The present invention contemplates therapeutic intervention of PKU/HPA. Such intervention is based initially on the use of BH4. The BH4 may be used alone or in combination with dietary restrictions. Further the BH4 and/or dietary restrictions may further be combined with other therapeutic compositions that are designed, for example to combat other manifestations of PKU, such as for example, large neutral amino acids to prevent Phe accumulation in the brain (see Koch et al., Mol. Genet. Metabol. 79:110-113, 2003) or tyrosine supplementation. The present section provides a discussion of the compositions that may be used in the treatments contemplated herein.

### A. BH4 Compositions

BH4 is a cofactor in Phe hydroxylation and prior to the present invention, it was shown that less than 2% of patients having an elevated Phe at birth have defects in BH4 synthesis. With those individuals that were identified as being BH4 responsive, it was suggested that the patients would be non-

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responsive to dietary intervention and hence, those individuals were fed a normal diet but given BH4 therapy alone. Thus, prior to the present invention, there was much skepticism in the art as to the therapeutic benefits of BH4 administration to PKU/HPA patients. However, as discussed herein throughout, BH4 may be administered for a therapeutic intervention of patients that have been diagnosed as non-BH4 responsive. Moreover, the present inventors show that BH4 therapy can be combined with dietary restrictions to produce a therapeutic outcome in both individuals that are responsive to a BH4 loading test as well as individuals that are non-responsive to BH4 loading.

U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408 and Canadian application CA 2420374 (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivative thereof that may be used as compositions for the present invention. Any such methods may be used to produce BH4 compositions for use in the therapeutic methods of the present invention.

U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment of PKU/HPA as described herein.

In addition to the above general methods of making BH4, the present invention particularly contemplates making and using a BH4 composition which is a stabilized BH4 composition. Preferably the stabilized BH4 composition is in crystalline form. Methods of making the stabilized BH4 compositions for use in the present invention are described in Example 2. Such a crystalline form may prove useful as an additive to conventional protein formulas for the treatment of PKU. The crystalline form also may conveniently be formed into a tablets, powder or other solid for oral administration. The forms and routes of administration of BH4 are discussed in further detail in the Pharmaceutical Compositions section below.

In preferred embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

Regardless of the amount of BH4 administered, it is desirable that the administration decreases the plasma Phe concentration of the patients to the concentrations discussed in Section I for the various types of patients.

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## B. Dietary Protein

In addition to administering BH4 and related analogs to HPA/PKU patients, it is contemplated that the dietary protein of the patients also may be restricted or modified. Those of skill in the art are aware of various commercially available protein formulas for use in the treatment of PKU. Such formulas include MAXIMAID, PHENEX 1, PHENEX 2 (Ross Laboratories, Liverpool, UK), LOFENALAC, PHENYL-FREE (Mead-Johnson), and the like.

Those of skill in the art may use the referenced protein formulas, which are generally free of Phe concentrations. The protein formulas often are supplemented with amino acids that are deficient in PKU patients. Such amino acids include, for example, L-tyrosine, and L-glutamine. It has been suggested that it may be desirable to supplement the diet of PKU patients with valine, isoleucine and leucine (see U.S. Pat. No. 4,252,822). In certain clinical manifestations, the toxic effects of PKU are caused by Phe blocking the brain uptake of other amino acids such as tyrosine and tryptophan. It has been found that supplementing the diet of a PKU patient with excess of such large neutral amino acids blocks Phe uptake into the brain and lowers brain Phe levels. Thus, it is contemplated that for the methods of the present invention, the dietary regimen may further be supplemented with compositions that comprise one or more of these amino acids (Koch et al., *Mol. Genet. Metabol.* 79:110-113, 2003).

Further, as it is known that L-carnitine and taurine which are normally found in human milk and other foodstuffs of animal origin also should be supplied in addition to the protein restriction. In certain embodiments, the L-carnitine may be supplied as 20 mg/100 g of protein supplement, and the taurine may be supplied as 40 mg/100 g protein supplement in order to help supply amounts of these factors normally found in human milk and foods of animal origin.

In addition, those of skill in the art are by reference to the 2000 National Academy of Sciences-National Research Council Dietary Reference Intakes for a further listing of other components, such as essential vitamins and minerals that should be supplied to the patient to ensure that other supplements are being provided despite the dietary protein restriction.

Referring to the Table presented in Section IC above for total protein amounts and the figures presented in Section I in general for the desirable plasma Phe concentrations, one of skill in the art will be able to determine the amount of dietary protein restriction that is required and thus adjust the diet of the patient accordingly. Taking for example, a male of about 11-14 years of age, that individual should preferably receive 45 g protein/day. In the event that the individual is one that has severe classic PKU, his unrestricted plasma Phe concentration will likely be greater than 1200  $\mu\text{M/L}$ , and most, if not all of the dietary protein source for that individual is likely to be from a powdered protein supplement, which preferably lowers his plasma Phe concentrations to less than 600  $\mu\text{M/L}$ . By administering BH4 to that subject, a therapeutic outcome would be one which produces greater decrease in the plasma Phe concentrations of patient or alternatively, the therapeutic outcome is one in which the individual's plasma Phe concentrations is lowered to a similar degree, but that individual is able to tolerate protein from a normal diet rather than from a dietary formula.

Similarly, a male of about 11-14 years of age, is one who has moderate PKU, it may be possible using the methods of the present invention to give him the allotted 45 g protein/day through a normal protein intake rather than a restricted formula. Determining whether the methods of the invention are effective will entail determining the plasma Phe concentra-

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tions of the patient on a regular basis to ensure that the plasma Phe concentrations remain below at least 400  $\mu\text{M/L}$ . Tests for determining such concentrations are described below. Preferably, concentrations of less than or about 360  $\mu\text{M/L}$  are achieved.

## III. IDENTIFYING AND MONITORING PATIENT POPULATIONS

As discussed herein throughout, it will be necessary in various embodiments of the present invention to determine whether a given patient is responsive to BH4 therapy, and to determine the phenylalanine concentrations of the patient both initially to identify the class of PKU patient being treated and during an ongoing therapeutic regimen to monitor the efficacy of the regimen. Exemplary such methods are described herein below.

## A. BH4 Loading Test

In order to identify a patient as being responsive to BH4, those of skill in the art perform a "BH4 loading" test. Two types of loading tests have been used to achieve the differential diagnosis of HPA. The first is a simple oral BH4 loading test and the second is a combined phenylalanine/BH4 loading test.

The simplest BH4 loading test is one in which exogenous BH4 is administered and the effects of the administration on lowering of plasma Phe concentrations is determined. Intravenous loading of 2 mg/kg BH4 was initially proposed by Danks et al., (*Lancet* 1:1236, 1976), as BH4 of greater purity has become available it has become possible to perform the test using an oral administration of BH4 in amounts of about 2.5 mg/kg body weight. Ultimately, a standardized approach was proposed by Niederwieser et al. in which a 7.5 mg/kg single oral dose of BH4 is administered (*Eur. J. Pediatr.* 138:441, 1982), although some laboratories do still use upwards of 20 mg BH4/kg body weight. This test allows discrimination between patients that have HPA due to a deficit in BH4 or through a deficiency in PAH.

In order for the simple BH4 loading test to produce reliable results, the blood Phe levels of the patient need to be higher than 400  $\mu\text{M/L}$ . Therefore, it is often customary for the patient to be removed from the PKU diet for 2 days prior to performing the loading test. A BH4 test kit is available and distributed by Dr. Schircks Laboratories (Jona, Switzerland). This kit recommends a dosage of 20 mg BH4/kg body weight about 30 minutes after intake of a normal meal.

As indicated above, the Phe concentration of a patient ideally needs to be higher than 400  $\mu\text{M/L}$  in order to obtain an accurate BH4 reading. In the combined Phenylalanine/BH4 loading test, an oral administration of Phe (100 mg/kg body weight) plus BH4 (20 mg/kg body weight) allows selective screening of all BH4 deficiencies. Typically, the Phe is administered in an oral dose and it is followed approximately one hour later with BH4. The plasma Phe levels are monitored before and at convenient time intervals (e.g., 1, 3, 5, 9, 13 and 25 hours) post-Phe administration.

In either the simple BH4 loading test or the combined Phe/BH4 loading test, it has been suggested that a decrease in plasma Phe of more than 30% of the plasma Phe value prior to BH4 challenge within 24 hours post-load is indicative of BH4 responsiveness (Spaapen et al, *Mol. Genet. and Metabol.*, 78:93-99, 2003).

Other methods of performing BH4 loading tests also may be used. Exemplary such tests are described in e.g., Muntau et al., (*N. Engl. J. Med.* 347(26):2002) and Bemeggar and Blau (*Mol. Genet. Metabol.* 77:304-313, 2002).



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In Bemeggar and Blau, the BH4 loading test uses 20 mg/kg BH4 and blood sampling for phenylalanine and tyrosine is performed at 0, 4, 8, and 24 hours to differentiate between BH4-responders and non-responders. The test is carried out after at least 3 hours of fasting. Urine samples of neopterin and bipterin are tested before the test. After an oral application of 6R BH4 (20 mg/kg body weight), normal food intake is allowed during the entire testing period. Blood samples are assayed for Phe and Tyr measurements at 0, 4, 8 and 24 hours. Another urine sample is collected between 4-8 hours. Dihydropteridine reductase activity also may be measured anytime during the test. In patients that have plasma phenylalanine levels less than 400  $\mu$ M/L or patients already on a low-phenylalanine diet, Bemeggar and Blau recommend a combined phenylalanine-BH4 test in which 100 mg Phe/kg body weight is administered orally 3 hours before the BH4 administration.

Bemeggar and Blau calculated BH4-responsiveness as "phenylalanine hydroxylation" at 4 and 8 hours after loading and was expressed as a percentage of the phenylalanine eliminated. The slope (S) of the graphs of "hydroxylation rates" at 0, 4 and 8 hours are compared for different BH4 products and different groups of patient. The slope discriminates between non-responders, slow responders and responders. The slow responders (see FIG. 5 in Bemeggar and Blau) need more time to reach the cut-off values of 360  $\mu$ M/L and that the effectiveness of administered BH4 depends on the initial phenylalanine levels. These authors recommend that for some patients with plasma Phe of less than 800  $\mu$ M/L and for most patients with a plasma Phe greater than 1200  $\mu$ M/L, a Phe measurement should be taken at 21 hours. A plot of Phe/S vs. time can be used to estimate the time needed to reach the therapeutic "safe" plasma Phe values of less than 360  $\mu$ M/L.

Muntau et al. (2002) also provide exemplary BH4 loading tests that can be used to calculate the times and concentrations of BH4 administration. Again these authors employed a combined PHE/BH4 loading test in which patients are given a meal that contains 100 mg Phe/kg body weight. One hour after the meal, the patients are given an oral dose of 20 mg/kg BH4 (Schirks Laboratories). Blood

The most popular method for determining Phe concentrations is the Guthrie test in which discs are punctured from filter paper that has been saturated with a blood sample from the patient. The uniform discs are incubated in a tray of agar that has been seeded with *Bacillus subtilis* and contains a specific inhibitor of *Bacillus subtilis* growth. As the phenylalanine transfers from the uniform discs onto the agar, the Phe reverse the inhibition of bacterial growth thereby yielding an area of bacterial growth that can be correlated to phenylalanine concentration by comparison to similar assays performed using discs containing known amounts of Phe.

Other methods of quantifying Phe concentration include HPLC, mass spectrometry, thin layer chromatography and the like. Such methods can be used to determine the plasma Phe concentration of a patient before the therapy and to monitor the Phe concentration during the therapeutic regimen to determine the efficacy thereof.

It is contemplated that the plasma Phe levels of the patients will be monitored at convenient intervals (e.g., daily, every other day or weekly) throughout the time course of the therapeutic regimen. By monitoring the plasma Phe levels with such regularity, the clinician will be able to assess the efficacy of the treatment and adjust the BH4 and/or dietary protein requirements accordingly.

#### IV. COMBINATION THERAPY

Certain methods of the invention involve the combined use of BH4 and dietary protein restriction to effect a therapeutic

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outcome in patients with various forms of HPA. To achieve the appropriate therapeutic outcome in the combination therapies contemplated herein, one would generally administer to the subject the BH4 composition and the dietary restriction in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4. The BH4 also may be formulated into a protein bar or other foodstuff such as brownies, pancakes, cake, suitable for ingestion.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, with a delay time of only about 1 hour being most preferred. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Gent. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4):243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

#### V. PHARMACEUTICAL COMPOSITIONS

Pharmaceutical compositions for administration according to the present invention can comprise a first composition

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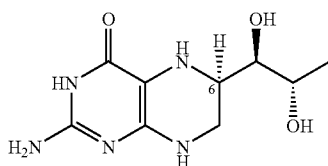
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comprising BH4 in a pharmaceutically acceptable form optionally combined with a pharmaceutically acceptable carrier. These compositions can be administered by any means that achieve their intended purposes. Amounts and regimens for the administration of a composition according to the present invention can be determined readily by those with ordinary skill in the art for treating PKU. As discussed above, those of skill in the art could initially employ amounts and regimens of BH4 currently being proposed in a medical context, e.g., those compositions that are being proposed for modulating NOS activity, or for use in the treatment of pain or depression as discussed in the patents listed in Section II above. Any of the protocols, formulations, routes of administration and the like described that have been used for administering BH4 for loading tests can readily be modified for use in the present invention.

The compositions and methods described herein are not limited to the use of a particular form of BH4, or form of an analog or derivative of BH4. Indeed, it is contemplated that the compositions and methods within the scope of this invention include all compositions comprising any form BH4, and any form of an analog or derivative thereof in an amount effective to achieve its intended purpose. Nonlimiting examples of analogs for use in the compositions and methods described herein include pteridine, pterin, neopterin, biop-  
terin, 7,8-Dihydrobiopterin, 6-methyltetrahydropterin, and other 6-substituted tetrahydropterin and other 6-substituted tetrahydropterins, sepiapterin, 6,7-Dimethyltetrahydropterin, 6-methyl biop-  
terin and other 6-substituted biop-  
terins, and other analogs that are described in the art. Nonlimiting examples of derivatives for use in the compositions and meth-  
ods described herein include the derivatives described in U.S. Pat. Nos. 4,758,571; 4,774,244; 6,162,806; 5,902, 810; 2,955,110; 2,541,717; 2,603,643; and 4,371,514, the disclo-  
sures of which are hereby incorporated herein.

Certain therapeutic methods of the present invention con-  
template a combination therapy in which BH4-based compo-  
sitions are administered in addition to a modified protein diet, the pharmaceutical compositions of the invention also con-  
template all compositions comprising at least BH4-based therapeutic agent, analog or homologue thereof in an amount effective to achieve the amelioration of one or more of the symptoms of PKU when administered in combination with the modified protein diet. Of course, the most obvious symp-  
tom that may be alleviated is that the combined therapy pro-  
duces a decrease in the plasma Phe concentration, however, other symptoms such as changes in IQ, executive function, concentration, mood, behavioral stability job performance and the like also may be monitored. Such indicia are moni-  
tored using techniques known to those of skill in the art. Crystalline Polymorphs of (6R) L-Tetrahydrobiopterin Dihy-  
drochloride Salt

It has been found that BH4, and in particular, the dihydro-  
chloride salt of BH4, exhibits crystal polymorphism. The structure of BH4 is shown below:



The (6R) form of BH4 is the known biologically active form, however, BH4 is also known to be unstable at ambient tem-

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peratures. It has been found that one crystal polymorph of BH4 is more stable, and is stable to decomposition under ambient conditions.

BH4 is difficult to handle and it is therefore produced and offered as its dihydrochloride salt (Schircks Laboratories, Jona, Switzerland) in ampoules sealed under nitrogen to pre-  
vent degradation of the substance due to its hygroscopic nature and sensitivity to oxidation. U.S. Pat. No. 4,649,197 discloses that separation of (6R)- and 6(S)-L-erythro-tetrahy-  
drobiopterin dihydrochloride into its diastereomers is diffi-  
cult due to the poor crystallinity of 6(R,S)-L-erythro-tetrahy-  
drobiopterin dihydrochloride. The European patent number 0 079 574 describes the preparation of tetrahydrobiopterin, wherein a solid tetrahydrobiopterin dihydrochloride is obtained as an intermediate. S. Matsuura et al. describes in Chemistry Letters 1984, pages 735-738 and Heterocycles, Vol. 23, No. 12, 1985 pages 3115-3120 6(R)-tetrahydrobiopterin dihydrochloride as a crystalline solid in form of color-  
less needles, which are characterized by X-ray analysis dis-  
closed in J. Biochem. 98, 1341-1348 (1985). An optical rotation of 6.81° was found the crystalline product, which is quite similar to the optical rotation of 6.51° reported for a crystalline solid in form of white crystals in example 6 of EP-A2-0 191 335.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride indicated that the compound may exist in different crystalline forms, including polymorphic forms and solvates. The continued interest in this area requires an efficient and reliable method for the preparation of the individual crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and controlled crystallization conditions to provide crystal forms, that are preferably stable and easy to handle and to process in the manufacture and preparation of formulations, and that provide a high storage stability in substance form or as formulated product, or which provide less stable forms suitable as intermediates for controlled crystallization for the manufacture of stable forms. Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B." Results obtained during investigation and development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride development revealed that there are several known crystalline solids have been prepared, but none have recognized the polymorphism and its effect on the stability of the BH4 crystals.

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application, which are prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

As used herein, the following the abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity. A characteristic X-ray powder diffraction pattern is exhibited in FIG. 1.

It has been found that other polymorphs of BH4 have a satisfactory chemical and physical stability for a safe handling during manufacture and formulation as well as providing a high storage stability in its pure form or in formulations. In addition, it has been found that form B, and other polymorphs of BH4 can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive crystal form B, can be used for the preparation of the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms than polymorph form B, such as polymorph A, in suitable polar and non aqueous solvents. Thus, the pharmaceutical preparations described herein refers to a preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Other forms of BH4 can be converted for form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methy-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallisation from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water:acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallisation from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallisation and isolation procedure can be advantageously carried out at

room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application.

#### Polymorph Form A

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form A," or "polymorph A." Polymorph A is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph A is a hygroscopic anhydrate which is a meta-stable form with respect to form B; however, it is stable over several months at ambient conditions if kept in a tightly sealed container. Form A is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form A can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Polymorph A which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) of: 15.5 (vs), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m), 3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w). FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph A exhibits a characteristic Raman spectra bands, expressed in wave numbers (cm<sup>-1</sup>) at: 2934 (w), 2880 (w), 1692 (s), 1683 (m), 1577 (w), 1462 (m), 1360 (w), 1237 (w), 1108 (w), 1005 (vw), 881 (vw), 813 (vw), 717 (m), 687 (m), 673 (m), 659 (m), 550 (w), 530 (w), 492 (m), 371 (m), 258 (w), 207 (w), 101 (s), 87 (s) cm<sup>-1</sup>.

Polymorph form A may be obtained by freeze drying or water removal of solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water. Polymorph form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be



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prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at ambient temperatures in water, (1) cooling the solution to low temperatures for solidifying the solution, and removing water under reduced pressure, or (2) removing water from said aqueous solution.

The crystalline form A can be isolated by filtration and then dried to evaporate absorbed water from the product. Drying conditions and methods are known and drying of the isolated product or water removal pursuant to variant (2) described herein may be carried out in applying elevated temperatures, for example up to 80° C., preferably in the range from 30° C. to 80° C., under vacuum or elevated temperatures and vacuum. Prior to isolation of a precipitate obtained in variant (2), the suspension may be stirred for a certain time for phase equilibration. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 5 to 40 percent by weight, by reference to the solution.

A fast cooling is preferred to obtain solid solutions as starting material. A reduced pressure is applied until the solvent is completely removed. Freeze drying is a technology well known in the art. The time to complete solvent removal is dependent on the applied vacuum, which may be from 0.01 to 1 mbar, the solvent used and the freezing temperature.

Polymorph form A is stable at room temperature or below room temperature under substantially water free conditions, which is demonstrated with phase equilibration tests of suspensions in tetrahydrofuran or tertiary-butyl methyl ether stirred for five days and 18 hours respectively under nitrogen at room temperature. Filtration and air drying at room temperature yields unchanged polymorph form A.

Polymorph Form F

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form F," or "polymorph F." Polymorph F is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph F is a meta-stable form and a hygroscopic anhydrate, which is more stable than form A at ambient lower temperatures and less stable than form B at higher temperatures and form F is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form F can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Polymorph F exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 17.1 (vs), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w), 3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 2.96 (m). FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph F may be obtained by phase equilibration of suspensions of polymorph form A in suitable polar and non-aqueous solvents, which scarcely dissolve said lower energy forms, especially alcohols such as methanol, ethanol, propanol and isopropanol. Polymorph form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can also be prepared by dispersing particles of solid form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-aqueous solvent that scarcely dissolves said (6R)-L-erythro-tetrahydrobiopterin dihydrochloride below room temperature, stirring the

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suspension at said temperatures for a time sufficient to produce polymorph form F, thereafter isolating crystalline form F and removing the solvent from the isolated form F. Removing of solvent and drying may be carried out under air, dry air or a dry protection gas such as nitrogen or noble gases and at or below room temperature, for example down to 0° C. The temperature during phase equilibration is preferably from 5 to 15° C. and most preferably about 10° C.

Polymorph Form J

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form J," or "polymorph J." The polymorph J is slightly hygroscopic and adsorbs water when handled at air humidity. The polymorph J is a meta-stable form and a hygroscopic anhydrate, and it can be transformed back into form E described below, from which it is obtained upon exposure to high relative humidity conditions such as above 75% relative humidity. Form J is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form J can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form J exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs). FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph J may be obtained by dehydration of form E at moderate temperatures under vacuum. In particular, polymorph form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by taking form E and removing the water from form E by treating form E in a vacuum drier to obtain form J at moderate temperatures which may mean a temperature in the range of 25 to 70° C., and most preferably 30 to 50° C.

Polymorph Form K

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form K," or "polymorph K." Polymorph K is slightly hygroscopic and adsorbs water to a content of about 2.0 percent by weight, which is continuously released between 50° C. and 100° C., when heated at a rate of 10° C./minute. The polymorph K is a meta-stable form and a hygroscopic anhydrate, which is less stable than form B at higher temperatures and form K is especially suitable as intermediate and starting material to produce stable polymorph forms, in particular form B. Polymorph form K can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form K exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m). FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph K may be obtained by crystallization from mixtures of polar solvents containing small amounts of water and in the presence of small amounts of ascorbic acid. Solvents for the solvent mixture may be selected from acetic acid

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and an alcohol such as methanol, ethanol, n- or isopropanol. In particular, polymorph form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and an alcohol or tetrahydrofuran containing small amounts of water and a small amount of ascorbic acid at elevated temperatures, lowering temperature below room temperature to crystallize said dihydrochloride, isolating the precipitate and drying the isolated precipitate at elevated temperature optionally under vacuum. Suitable alcohols are for example methanol, ethanol, propanol and isopropanol, whereby ethanol is preferred. The ratio of acetic acid to alcohol or tetrahydrofuran may be from 2:1 to 1:2 and preferably about 1:1. Dissolution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be carried out in presence of a higher water content and more of the anti-solvent mixture can be added to obtain complete precipitation. The amount of water in the final composition may be from 0.5 to 5 percent by weight and the amount of ascorbic acid may be from 0.01 to 0.5 percent by weight, both by reference to the solvent mixture. The temperature for dissolution may be in the range from 30 to 100 and preferably 35 to 70° C. and the drying temperature may be in the range from 30 to 50° C. The precipitate may be washed with an alcohol such as ethanol after isolation, e.g., filtration. The polymorph K can easily be converted in the most stable form B by phase equilibration in e.g., isopropanol and optionally seeding with form B crystals at above room temperature such as temperatures from 30 to 40° C.

**Hydrate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt**

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline hydrate, which shall be described and defined herein as forms C, D, E, H, and O. These hydrate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

**Hydrate Form C**

It has been found that a hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form C," or "hydrate C." The hydrate form C is slightly hygroscopic and has a water content of approximately 5.5 percent by weight, which indicates that form C is a monohydrate. The hydrate C has a melting point near 94° C. ( $\Delta H_f$  is about 31 J/g) and hydrate form C is especially suitable as intermediate and starting material to produce stable polymorphic forms. Polymorph form C can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form C exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m). FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form C may be obtained by phase equilibration at ambient temperatures of a polymorph form such as polymorph B suspension in a non-solvent which contains water in an amount of preferably about 5 percent by weight, by reference to the solvent. Hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by sus-

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sending (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-solvent such as, heptane, C1-C4-alcohols such as methanol, ethanol, 1- or 2-propanol, acetates, such as ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or binary or ternary mixtures of such non-solvents, to which sufficient water is added to form a monohydrate, and stirring the suspension at or below ambient temperatures (e.g., 0 to 30° C.) for a time sufficient to form a monohydrate. Sufficient water may mean from 1 to 10 and preferably from 3 to 8 percent by weight of water, by reference to the amount of solvent. The solids may be filtered off and dried in air at about room temperature. The solid can absorb some water and therefore possess a higher water content than the theoretical value of 5.5 percent by weight. Hydrate form C is unstable with respect to forms D and B, and easily converted to polymorph form B at temperatures of about 40° C. in air and lower relative humidity. Form C can be transformed into the more stable hydrate D by suspension equilibration at room temperature.

**Hydrate Form D**

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form D," or "hydrate D." The hydrate form D is slightly hygroscopic and may have a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form D is a monohydrate. The hydrate D has a melting point near 153° C. ( $\Delta H_f$  is about 111 J/g) and is of much higher stability than form C and is even stable when exposed to air humidity at ambient temperature. Hydrate form D can therefore either be used to prepare formulations or as intermediate and starting material to produce stable polymorph forms. Polymorph form D can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form D exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w). FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form D may be obtained by adding at about room temperature concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents, and stirring the suspension at ambient temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding at about room temperature a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. Form D contains a small excess of water, related to the monohydrate, and it is believed that it is absorbed water due to the slightly hygroscopic nature of this crystalline

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hydrate. Hydrate form D is deemed to be the most stable one under the known hydrates at ambient temperatures and a relative humidity of less than 70%. Hydrate form D may be used for formulations prepared under conditions, where this hydrate is stable. Ambient temperature may mean 20 to 30° C. Hydrate Form E

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form E,” or “hydrate E.” The hydrate form E has a water content of approximately 10 to 14 percent by weight, which suggests that form E is a dihydrate. The hydrate E is formed at temperatures below room temperature. Hydrate form E is especially suitable as intermediate and starting material to produce stable polymorph forms. It is especially suitable to produce the water-free form J upon drying under nitrogen or optionally under vacuum. Form E is non-hygroscopic and stable under rather high relative humidities, i.e., at relative humidities above about 60% and up to about 85%. Polymorph form E can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form E exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w). FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form E may be obtained by adding concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent cooled to temperatures from about 10 to -10° C. and preferably between 0 to 10° C. and stirring the suspension at said temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent which is cooled to temperatures from about 10 to -10° C., and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. A preferred non-solvent is tetrahydrofuran. Another preparation process comprises exposing polymorph form B to an air atmosphere with a relative humidity of 70 to 90%, preferably about 80%. Hydrate form E is deemed to be a dihydrate, whereby some additional water may be absorbed. Polymorph form E can be transformed into polymorph J upon drying under vacuum at moderate temperatures, which may mean between 20° C. and 50° C. at pressures between 0 and 100 mbar. Form E is especially suitable for formulations in semi solid forms because of its stability at high relative humidities.

Hydrate Form H

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical

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preparation described herein, which shall be referred to herein as “form H,” or “hydrate H.” The hydrate form H has a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form H is a hygroscopic monohydrate. The hydrate form H is formed at temperatures below room temperature. Hydrate form H is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form H can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form H exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 15.8 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w), 3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m). FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form H may be obtained by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water, adding then a non-solvent to precipitate a crystalline solid, cooling the obtained suspension and stirring the cooled suspension for a certain time. The crystalline solid is filtered off and then dried under vacuum at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is tetrahydrofuran. Hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and a less amount than that of acetic acid of water, adding a non-solvent and cooling the obtained suspension to temperatures in the range of -10 to 10° C., and preferably -5 to 5° C., and stirring the suspension at said temperature for a certain time. Certain time may mean 1 to 20 hours. The weight ratio of acetic acid to water may be from 2:1 to 25:1 and preferably 5:1 to 15:1. The weight ratio of acetic acid/water to the non-solvent may be from 1:2 to 1:5. Hydrate form H seems to be a monohydrate with a slight excess of water absorbed due to the hygroscopic nature.

Hydrate Form O

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as “form O,” or “hydrate O.” The hydrate form O is formed at temperatures near room temperature. Hydrate form O is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form O can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form O exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m). FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.



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Hydrate form O can be prepared by exposure of polymorphic form F to a nitrogen atmosphere containing water vapor with a resulting relative humidity of about 52% for about 24 hours. The fact that form F, which is a slightly hygroscopic anhydrate, can be used to prepare form O under 52% relative humidity suggests that form O is a hydrate, which is more stable than form F under ambient temperature and humidity conditions.

Solvate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline solvate forms, which shall be described and defined herein as forms G, I, L, M, and N. These solvate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

Solvate Form G

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form G," or "hydrate G." The ethanol solvate form G has a ethanol content of approximately 8.0 to 12.5 percent by weight, which suggests that form G is a hygroscopic mono ethanol solvate. The solvate form G is formed at temperatures below room temperature. Form G is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form G can be prepared as a solid powder with a desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form G exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w). FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form G may be obtained by crystallization of L-erythro-tetrahydrobiopterin dihydrochloride dissolved in water and adding a large excess of ethanol, stirring the obtained suspension at or below ambient temperatures and drying the isolated solid under air or nitrogen at about room temperature. Here, a large excess of ethanol means a resulting mixture of ethanol and water with less than 10% water, preferably about 3 to 6%. Ethanolate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at about room temperature to temperatures of 75° C. (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water or in a mixture of water and ethanol, cooling a heated solution to room temperature and down to 5 to 10° C., adding optionally ethanol to complete precipitation, stirring the obtained suspension at temperatures of 20 to 5° C., filtering off the white, crystalline solid and drying the solid under air or a protection gas such as nitrogen at temperatures about room temperature. The process may be carried out in a first variant in dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at about room temperature in a lower amount of water and then adding an excess of ethanol and then stirring the obtained suspension for a time sufficient for phase equilibration. In a second variant, (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be suspended in ethanol, optionally adding a lower amount of water, and heating the suspension and dissolute (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, cooling down the solution to temperatures

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of about 5 to 15° C., adding additional ethanol to the suspension and then stirring the obtained suspension for a time sufficient for phase equilibration.

Solvate Form I

It has been found that an acetic acid solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form I," or "hydrate I." The acetic acid solvate form I has an acetic acid content of approximately 12.7 percent by weight, which suggests that form I is a hygroscopic acetic acid mono solvate. The solvate form I is formed at temperatures below room temperature. Acetic acid solvate form I is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form I can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form I exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m), 3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w). FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Acetic acid solvate form I may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water at elevated temperature, adding further acetic acid to the solution, cooling down to a temperature of about 10° C., then warming up the formed suspension to about 15° C., and then stirring the obtained suspension for a time sufficient for phase equilibration, which may last up to 3 days. The crystalline solid is then filtered off and dried under air or a protection gas such as nitrogen at temperatures about room temperature.

Solvate Form L

It has been found that a mixed ethanol solvate/hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form L," or "hydrate L." Form L may contain 4% but up to 13% ethanol and 0% to about 6% of water. Form L may be transformed into form G when treated in ethanol at temperatures from about 0° C. to 20° C. In addition form L may be transformed into form B when treated in an organic solvent at ambient temperatures (10° C. to 60° C.). Polymorph form L can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form L exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w). FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Form L may be obtained by suspending hydrate form E at room temperature in ethanol and stirring the suspension at temperatures from 0 to 10° C., preferably about 5° C., for a time sufficient for phase equilibration, which may be 10 to 20 hours. The crystalline solid is then filtered off and dried preferably under reduced pressure at 30° C. or under nitrogen. Analysis by TG-FTIR suggests that form L may contain

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variable amounts of ethanol and water, i.e., it can exist as an polymorph (anhydrate), as a mixed ethanol solvate/hydrate, or even as a hydrate.

## Solvate Form M

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form M," or "hydrate M." Form M may contain 4% but up to 13% ethanol and 0% to about 6% of water, which suggests that form M is a slightly hygroscopic ethanol solvate. The solvate form M is formed at room temperature. Form M is especially suitable as intermediate and starting material to produce stable polymorph forms, since form M can be transformed into form G when treated in ethanol at temperatures between about -10° to 15° C., and into form B when treated in organic solvents such as ethanol, C3 and C4 alcohols, or cyclic ethers such as THF and dioxane. Polymorph form M can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form M exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs). FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in ethanol and evaporation of the solution under nitrogen at ambient temperature, i.e., between 10° C. and 40° C. Form M may also be obtained by drying of form G under a slight flow of dry nitrogen at a rate of about 20 to 100 ml/min. Depending on the extent of drying under nitrogen, the remaining amount of ethanol may be variable, i.e., from about 3% to 13%.

## Solvate Form N

It has been found that another solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form N," or "hydrate N." Form N may contain in total up to 10% of isopropanol and water, which suggests that form N is a slightly hygroscopic isopropanol solvate. Form N may be obtained through washing of form D with isopropanol and subsequent drying in vacuum at about 30° C. Form N is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form N can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Form N exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83(w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w). FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

The isopropanol form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in 4.0 ml of a mixture of isopropanol and water (mixing volume ratio for example 4:1). To this solution is slowly added isopropanol (IPA, for example about 4.0 ml) and the resulting suspension is cooled to 0° C. and stirred for several hours (e.g., about 10 to 18 hours) at this temperature. The suspension is filtered and the solid residue washed with isopropanol at room temperature. The obtained crystalline material is then dried at ambient temperature (e.g., about 20 to 30° C.) and reduced pressure (about 2 to 10 mbar) for several hours (e.g., about 5 to 20

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hours). TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water. This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as a non-solvated form containing a small amount of water.

For the preparation of the polymorph forms, there may be used crystallization techniques well known in the art, such as stirring of a suspension (phase equilibration in), precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 100° C. may be applied to form solutions. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Meta-stable polymorphs or pseudo-polymorphic forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

It was surprisingly found that hydrate form D is the most stable form under the hydrates and forms B and D are especially suitable to be used in pharmaceutical formulations. Forms B and D presents some advantages like an aimed manufacture, good handling due to convenient crystal size and morphology, very good stability under production conditions of various types of formulation, storage stability, higher solubility, and high bio-availability. Accordingly, in a method and/or a composition disclosed herein the form of BH4 present in a mixture is preferably a stabilized crystal form of BH4 an is selected from the group consisting of crystal polymorph form A, crystal polymorph form B, crystal polymorph form F, crystal polymorph form J, crystal polymorph form K, crystal hydrate form C, crystal hydrate form D, crystal hydrate form E, crystal hydrate form H, crystal hydrate form O, solvate crystal form G, solvate crystal form I, solvate crystal form L, solvate crystal form M, solvate crystal form N, and combinations thereof. More preferably, the form of BH4 is for use in a composition and method disclosed herein is pharmaceutical composition including polymorph form B and/or hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

The crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be used together with folic acid or tetrahydrofolic acid or their pharmaceutically acceptable salts such as sodium, potassium, calcium or ammonium salts, each alone or additionally with arginine. The weight ratio of crystal forms:folic acids or salts thereof:arginine may be from about 1:10:10 to about 10:1:1.

## VI. PHARMACEUTICAL FORMULATIONS

The formulations described herein are preferably administered as oral formulations. Oral formulations are preferably solid formulations such as capsules, tablets, pills and troches, or liquid formulations such as aqueous suspensions, elixirs and syrups. The various form of BH4 described herein can be directly used as powder (micronized particles), granules, suspensions or solutions, or it may be combined together with other pharmaceutically acceptable ingredients in admixing the components and optionally finely divide them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches, or suspend or dissolve them in carriers for suspensions, elixirs and syrups. Coatings may be applied after compression to form pills.

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Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be for example binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. Nonlimiting examples of binders useful in a composition described herein include gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (polyoxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Nonlimiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate. Nonlimiting examples of lubricants use in a composition described herein include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Surfactants for use in a composition described herein can be anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The hydrate form D described herein may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. A syrup or elixir may contain the polymorph described herein,

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sucrose or fructose as sweetening agent a preservative like methylparaben, a dye and a flavoring agent.

Slow release formulations may also be prepared from the polymorph described herein in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg×5 kg body weight) to 3000 mg/day (30 mg/kg×100 kg body weight). Such a dose may be administered in a single dose or it may be divided into multiple doses. While continuous, daily administration is contemplated, it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

As discussed above, the total dose required for each treatment may be administered in multiple doses or in a single dose. The BH4 and the protein compositions may be administered alone or in conjunction with other therapeutics directed to the disease or directed to other symptoms thereof.

As is apparent from the disclosure presented herein, in a broad aspect the present application contemplates clinical application of a combination therapy comprising a first composition that contains a crystallized BH4 formulation, and a second composition that contains a medical protein formulation (e.g., PHENEX or the like). Therefore, the compositions should be formulated into suitable pharmaceutical compositions, i.e., in a form appropriate for in vivo applications in such combination therapies. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals. Preferably, the crystallized BH4 composition may be such that it can be added directly to the preexisting protein formulations used for the treatment of PKU.

One will generally desire to employ appropriate salts and buffers to render the BH4 suitable for uptake. Aqueous compositions of the present invention comprise an effective amount of the BH4 dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions may be administered orally or via injection.



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The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions. In exemplary embodiments, the medical protein formulation may comprise corn syrup solids, high-oleic safflower oil, coconut oil, soy oil, L-leucine, calcium phosphate tribasic, L-tyrosine, L-proline, L-lysine acetate, DATEM (an emulsifier), L-glutamine, L-valine, potassium phosphate dibasic, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-serine, potassium citrate, L-threonine, sodium citrate, magnesium chloride, L-histidine, L-methionine, ascorbic acid, calcium carbonate, L-glutamic acid, L-cystine dihydrochloride, L-tryptophan, L-aspartic acid, choline chloride, taurine, m-inositol, ferrous sulfate, ascorbyl palmitate, zinc sulfate, L-carnitine, alpha-tocopheryl acetate, sodium chloride, niacinamide, mixed tocopherols, calcium pantothenate, cupric sulfate, thiamine chloride hydrochloride, vitamin A palmitate, manganese sulfate, riboflavin, pyridoxine hydrochloride, folic acid, beta-carotene, potassium iodide, phyloquinone, biotin, sodium selenate, chromium chloride, sodium molybdate, vitamin D3 and cyanocobalamin. The amino acids, minerals and vitamins in the supplement should be provided in amounts that provide the recommended daily doses of each of the components.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention include classic pharmaceutical preparations of BH4 which have been discussed herein as well as those known to those of skill in the art. Protein formulas, such as, e.g., Phenex, also are known to those of skill in the art. Administration of these compositions according to the present invention will be via any common route for dietary supplementation. The protein is preferably administered orally, as is the BH4.

The active compositions may be prepared for administration as solutions of free base or pharmacologically acceptable salts in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The BH4 compositions may be prepared as pharmaceutical forms suitable for injectable use. Such compositions include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of

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microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used.

Compositions and methods for producing a stabilized tablet formulation are also disclosed in co-pending U.S. provisional application No. 60/629,189 filed Nov. 17, 2004, the entirety of which is hereby incorporated by reference.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N' dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4

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aminosalicylic acid, 2 phenoxybenzoic acid, 2 acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzenesulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2 sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data. The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus the subject to be treated may be a mammal, preferably human or other animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkey ducks and geese.

## VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be

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made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Clinical Evaluation with 6R-Tetrahydrobiopterin

The following example provides guidance on the parameters to be used for the clinical evaluation BH4 in the therapeutic methods of the present invention. As discussed herein throughout, BH4 will be used in the treatment of HPA including HPA, mild phenylketonuria (PKU) and classic PKU. Clinical trials will be conducted which will provide an assessment of daily oral doses of BH4 for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum, but not necessarily limited to, 6 weeks to collect sufficient safety information for 30 evaluable patients.

The initial dose for the trials will vary from about 10 to about 20 mg/kg. In the event that this dose does not produce a reduction in excess plasma phenylalanine (Phe) levels in a patient, or produce a significant direct clinical benefit measured as an ability to increase daily oral Phe intake without increases in plasma Phe levels, the dose should be increased as necessary, and maintained for an additional minimal period of, but necessarily limited to, 6 weeks to establish safety and to evaluate further efficacy. Lower doses, e.g., doses of between 5 to 10 mg/kg also are contemplated.

Measurements of safety will include adverse events, allergic reactions, complete clinical chemistry panel (kidney and liver function), urinalysis, and CBC with differential. In addition, other parameters including the reduction in levels of blood Phe levels, neuropsychological and cognitive testing, and global assessments also will be monitored. The present example also contemplates the determination of pharmacokinetic parameters of the drug in the circulation, and general distribution and half-life of 6R-BH4 in blood. It is anticipated that these measures will help relate dose to clinical response.

### Methods

Patients who have elevated levels of plasma Phe will undergo a baseline a medical history and physical exam, neuropsychological and cognitive testing, a standard set of clinical laboratory tests (CBC, Panel 20, CH50, UA), levels of urinary pterins, dihydropteridine reductase (DHPR) levels, and a fasting blood (plasma) panel of serum amino acids. The proposed human dose of 10 to about 20 mg/kg BH4 will be administered divided in one to three daily doses. The patient will be followed closely with weekly visits to the clinic. Patients will return to the clinic for a complete evaluation one week after completing the treatment period. Should dose escalation be required, the patients will follow the same schedule outlined above. Safety will be monitored throughout the trial.

Enrolled patients will be randomized to receive BH4 or a placebo. After an initial two to four-week period all study participants will be placed on a controlled diet with a limited Phe intake for a total of four to six weeks. After completing the first two to four weeks on dietary restriction, all study participants will be crossed-over in their randomization and will followed for an additional two to four weeks. The blood levels and other biochemical parameters will be followed closely at the end of each period. Evaluation of neuropsychological outcomes will include measurements of sustained attention; working memory; and ability to perform complex

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operations. Patients who complete the trial, and who benefited from therapy by showing a beneficial decrease plasma Phe levels, will be offered continued BH4 therapy thorough an extended protocol for as long as safety and efficacy conditions warrant it, or until BLA approval.

#### Diagnosis and Inclusion/Exclusion Criteria

The patient may be male or female, aged twelve years or older with a documented diagnosis of HPA or mild PKU confirmed by genetic testing and evidence of elevated Phe levels in blood. The study will include HPA or PKU patients who do not accurately follow dietary control. Female patients of childbearing potential must have a negative pregnancy test (urine  $\beta$ -hCG) just prior to each dosing and must be advised to use a medically accepted method of contraception throughout the study. A patient will be excluded from this study if the patient has evidence of a primary BH4 deficiency, has previously received multiple doses of BH4 for more than 1 week of treatment; is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

#### Dose, Route and Regimen

Patients will receive BH4 at a dose of 5-10 mg/kg per day. In the event that Phe blood levels are not decreased by a reasonable amount and no clinical benefit is observed, the dose will be increased as necessary. Dose escalation will occur only after all patients have undergone at least 2 weeks of therapy. The daily BH4 dosage will be administered orally as liquid, powder, tablets or capsules. The total daily dose may be given as a single dose or perhaps divided in two or three daily doses. The patients will be monitored clinically as well as for any adverse reactions. If any unusual symptoms are observed, study drug administration will be stopped immediately, and a decision will be made about study continuation.

#### Dietary Intervention

Following the initial randomization and two-week treatment period, all study participants will undergo dietary counseling and will follow a standard Phe-restricted diet complemented with Phe-specific medical foods for a total of four to six weeks. Diets will be managed at home and dietary intake will be recorded in daily logs. Analyses of the intakes of nutrients and medical foods and the percent of Recommended Dietary Intakes (RDI) will be compared among the treatment groups.

#### BH4 Safety

BH4 therapy will be determined to be safe if no significant acute or chronic drug reactions occur during the course of the study. The longer-term administration of the drug will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies.

#### Example 2

##### Preparation of Stabilized Crystallized form of BH4

U.S. Provisional Patent Application Ser. No. 60/520,377, entitled "Polymorphs of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride" filed on Nov. 17, 2003 in the name of Applicants Rudolf MOSER, of Schaffhausen, Switzerland and Viola GROEHN of Dachsen, Switzerland and assigned Merck-Eprova internal reference number 216, and U.S. patent application Ser. No. 10/990,316, entitled "Polymorphs of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride" filed concurrently herewith on Nov. 17, 2004 in the name of Appli-

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cants Rudolf MOSER, of Schaffhausen, Switzerland and Viola GROEHN of Dachsen, Switzerland and assigned Merck-Eprova internal reference number 216/US CIP (both of the Moser et al. applications are collectively referred to herein as the "Moser Applications" and both are incorporated herein by reference in their entireties. The examples of that specification describe X ray and Raman spectra studies to characterize the polymorphs of BH4. Each of the BH4 compositions of that application may be used in the treatment methods described herein. The following description provides additional background and a brief characterization of some of those exemplary compositions.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride (see the Moser Applications) indicated that the compound may possess polymorphic forms. The continued interest in this area requires an efficient and reliable method for the preparation of individual polymorphs of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and controlled crystallization conditions to provide polymorphs, which are preferably stable and easily to handle and to process in the manufacture and preparation of formulations.

Crystallization techniques well known in the art for producing drug crystals are used to prepare the polymorph forms. Such techniques include, but are not limited to, techniques such as suspension, precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions of the BH4 may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 150° C. may be applied to form solutions of the drug. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Metastable polymorph or pseudo-polymorph forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

As discussed in the Moser Applications, the polymorph form may be obtained by crystallization of the BH4 from polar solvent mixtures. The Moser Applications also describes a process for the preparation of polymorph form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolution, optionally at elevated temperatures, of a solid lower energy form than the claimed form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a polar solvent mixture, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals.

Dissolution may be carried out at room temperature or up to 70° C., More preferably the dissolution is carried out at temperatures up to 50° C. The starting material may be added to the final solvent mixture for dissolution, or alternatively the starting material first may be dissolved in water and other solvents may than be added both or one after the other solvent. The solution of the BH4 is preferably stirred. Cooling may mean temperatures down to -80° C., preferably down to -40° C. to 0° C. In some embodiments, in order to initiate the crystallization of the BH4 polymorph, the solution may be seeded. Suitable seeds may include a portion of the polymorph form from another batch of crystals, or crystals having a similar or identical morphology. After isolation, the crystalline form can be washed with acetone or tetrahydrofuran and dried using techniques commonly used for drying drug crystals.

The polymorph forms of BH4 described in the Moser Applications are a very stable crystalline form of the drug. The polymorph form can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formula-



tions. These outstanding properties renders this polymorph form especially feasible for pharmaceutical application. The stability of the polymorph form of BH4 was determined after the BH4×2HCl (the polymorph form) had been stored for 8 months in a minigrip bag at 40° C. and 75% relative humidity. Quality was checked in different intervals throughout the 8 month period by HPLC. After 8 months, the quality and stability of the polymorph was surprisingly similar to the stability seen at time zero:

	0 months (at the beginning)	after 1 week	after 1 month	after 3 months	after 8 months
HPLC [% area]	98.4	99.4	98.3	99.1	98.1

Accordingly, the Moser Applications provides descriptions of a pharmaceutical compositions comprising a polymorph form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent. Such compositions will be useful in the therapeutic methods described herein.

In addition to the Moser Applications, those of skill in the art also are referred to U.S. Pat. Nos. 6,596,721; 6,441,168; and 6,271,374 which describe various methods and compositions for producing stable crystalline salts of 5-methyltetrahydrofolic acid and methods and compositions for producing stable forms of 6R tetrahydrofolic acid and methods and compositions for producing stable forms of 6S and 6R tetrahydrofolic acid. Each of these patents are incorporated herein by reference in their entirety as generally teaching methods of producing crystalline forms of agents and techniques for characterizing such agents. Such methods may be used in producing stable forms of BH4 for use as pharmaceutical compositions in the treatment methods taught herein.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

### Example 3

#### Administration of Tetrahydrobiopterin to Humans with Elevated Serum Phe Levels

An open label, single and multiple dose study was conducted in a total of 20 patients to demonstrate the safety and efficacy of tetrahydrobiopterin in humans with elevated blood levels of phenylalanine (>600 µmol/L). Criteria for inclusion in the study included (1) baseline blood Phe levels of >600 µmol/L, (2) age of at least 8 years. Criteria for exclusion from the study included (1) pregnancy or breastfeeding, (2) concurrent diseases or conditions that require medication or

treatment, (3) concurrent treatment with any drug known to inhibit folate synthesis, and (4) treatment with any investigational drug within 30 days. Each of the patients also was identified as having a mutation in the phenylalanine hydroxylase (PAH) gene. Study subjects underwent baseline assessments, including medical history with assessment of phenylketonuria (PKU) or hyperphenylalaninemia (HPA) related signs and symptoms, physical examinations, vital signs, serum amino acid (i.e., phenylalanine, tyrosine, and tryptophan) blood levels, and routine laboratory tests (chemistry, hematology, and urinalysis) before inclusion in the study.

The drug tested was (6R)-5,6,7,8-tetrahydrobiopterin, also known as 2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-3H-pteridin-4-one tetrahydrobiopterin, or sapropterin (BH4 or 6R-BH4. The drug was obtained in 10 mg or 50 mg oral tablets from Schircks Laboratories, Switzerland (product no. 11.212 (6R)-5,6,7,8-Tetrahydro-L-biopterin dihydrochloride). The half-life of the Schircks 6R-BH4 dihydrochloride salt is approximately 3.5 hours.

Drugs known to inhibit folate synthesis such as bactrim, methotrexate, or 5-FU were not permitted to be administered during the study. Before initiation of 6R-BH4 dosing, a 7 day washout period was required for any drugs known to inhibit folate synthesis. No investigational drugs were permitted to be taken during study participation or within 30 days prior to study enrollment.

Within a maximum of 4 weeks following the completion of baseline assessments, eligible subjects began the first stage of the study. Single ascending doses of 10 mg/kg, 20 mg/kg and 40 mg/kg of 6R-BH4 were administered orally, with a washout period of at least 7 days between each dose, and subjects were monitored 24 hours after each dose. Subjects underwent a safety assessment and blood amino acid (i.e., phenylalanine, tyrosine, and tryptophan) level measurements before and 24 hours after each 6R-BH4 dose. Blood pressure was measured 30 minutes and 1 hour after each dose. Safety assessments included physical examinations, vital signs, serial assessment of PKU or HPA related signs and symptoms, recording of adverse events, and monitoring of changes in laboratory parameters (chemistry, hematology, and urinalysis). Subjects were instructed to continue their usual diet without any modification, and to record daily intake of food and beverages throughout the study.

After the first stage of the study was completed, subjects entered the second stage of the study, during which they received 10 mg/kg of 6R-BH4 daily in an oral dosage form, for a total of 7 days. After a washout period of at least 7 days, each subject received 20 mg/kg of 6R-BH4 daily for a total of 7 days. During the second stage of the study, subjects were monitored before dosing, at 24 and 72 hours after first dose, and on the 7th day of dosing at each of the two dose levels. Monitoring included a safety assessment as described above, measurement of serum blood amino acid (i.e., phenylalanine, tyrosine, and tryptophan) levels and evaluation of phenylalanine and tyrosine oral intake. Subjects were instructed to continue their usual diet without any modification, and to record daily intake of food and beverages throughout the study.

After a single dose of 6R-BH4 (10 mg/kg), blood Phe declined 10%±0.26% from baseline. Single doses of 6R-BH4 at 20 mg/kg and 40 mg/kg showed mean declines of 17%±0.28% and 27%±0.25% respectively. The reduction in blood Phe levels appeared to be dose dependent.

FIG. 16 shows mean blood phenylalanine level after 10 and 20 mg/kg 6R-BH4 daily for 7 days, in the 14 of 20 patients who responded to treatment. For the purposes of this study, a decline in blood Phe levels of 30% was considered to be

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“responsive”, although patients who exhibit less of a decline would still benefit from BH4 treatment. The seven-day trial showed a sustained decrease in blood Phe concentration in 70% of the patients (14/20) taking 20 mg/kg. Of those 14 patients, 10 (71%) responded favorably to 10 mg/kg/day. Blood tyrosine was observed to increase in some but not all patients; some patients had increases of >80% from baseline tyrosine levels. The individual blood Phe responses to multiple doses of 10 mg/kg BH4 are shown in 11 adults (FIG. 17) and 9 children (FIG. 19). The individual blood Phe responses to multiple doses of 20 mg/kg BH4 are shown in 11 adults (FIG. 18) and in 9 children (FIG. 20).

Thus, a single-dose loading test was inadequate to identify patients who responded to BH4 treatment with a reduction in blood Phe level of 30% or more. A 7-day loading test successfully identified a high percentage of responsive patients. The 20 mg/kg, 7-day loading test with 6R-BH4 identified 70% of the PKU patients that responded to 20 mg/kg of BH4. Of the 14 responders, 71% also showed a 30% or greater reduction in blood Phe level with the lower dose of 10 mg/kg 6R-BH4.

The references cited herein throughout, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are all specifically incorporated herein by reference.

What is claimed is:

1. A method for treating a subject with hyperphenylalaninemia comprising administering to said subject a therapeutically effective amount of tetrahydrobiopterin (BH4) or pharmaceutically acceptable salt thereof, wherein the BH4 is administered orally once per day.

2. The method of claim 1, wherein the hyperphenylalaninemia results from BH4 deficiency.

3. The method of claim 1, wherein the hyperphenylalaninemia results from phenylketonuria (PKU).

4. The method of claim 1, 2 or 3, wherein said subject is administered BH4 for at least 7 days.

5. The method of claim 1, 2 or 3, wherein the subject is administered BH4 for at least 2 weeks.

6. The method of claim 1, 2 or 3, wherein said subject is administered BH4 for at least 6 weeks.

7. The method of claim 1, wherein said subject has a plasma phenylalanine concentration of greater than 180  $\mu$ M prior to treatment with BH4.

8. The method of claim 1, wherein said subject has a plasma phenylalanine concentration of greater than 600  $\mu$ M prior to treatment with BH4.

9. The method of claim 1, wherein said subject has a plasma phenylalanine concentration of greater than 1000  $\mu$ M prior to treatment with BH4.

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10. The method of claim 1, wherein said subject has a plasma phenylalanine concentration of greater than 1200  $\mu$ M prior to treatment with BH4.

11. The method of claim 1, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 600  $\mu$ M.

12. The method of claim 1, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 500  $\mu$ M.

13. The method of claim 1, wherein said administration of BH4 decreases the plasma phenylalanine concentration of said subject to less than 360  $\mu$ M.

14. The method of claim 1, wherein said BH4 is administered in a daily dose of between about 1 mg/kg to about 30 mg/kg.

15. The method of claim 1, wherein said BH4 is administered in a daily dose of between about 5 mg/kg to about 30 mg/kg.

16. The method of claim 1, wherein said BH4 is administered as a crystallized form stable for at least 3 months at 40° C. and 75% relative humidity.

17. The method of claim 16, wherein said crystallized form of BH4 comprises at least 99.5% pure (6R)-5,6,7,8-tetrahydrobiopterin.

18. The method of claim 16, wherein said crystallized form of BH4 comprises purified polymorph B, wherein polymorph B, as a hydrochloride salt, exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 5.63 (m), 4.76(m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), and 2.44 (w).

19. The method of claim 1, further comprising administering to said subject a protein-restricted diet.

20. The method of claim 19, wherein said protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 600 mg per day.

21. The method of claim 19, wherein said protein-restricted diet is a phenylalanine-restricted diet wherein the total phenylalanine is restricted to less than 300 mg per day.

22. The method of claim 19, wherein said subject is a pregnant female.

23. The method of claim 19, wherein said subject is an infant between the ages of 0 and 3 years of age.

24. The method of claim 19, wherein said subject is a female of child-bearing age that is contemplating pregnancy.

\* \* \* \* \*

# **EXHIBIT F**





US00RE43797E

(19) **United States**  
 (12) **Reissued Patent**  
**Oppenheimer et al.**

(10) **Patent Number:** **US RE43,797 E**  
 (45) **Date of Reissued Patent:** **\*Nov. 6, 2012**

(54) **METHODS OF ADMINISTERING  
TETRAHYDROBIOPTERIN**

(75) Inventors: **Daniel I. Oppenheimer**, Castro Valley,  
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**Okhamafe**, Concord, CA (US)

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(\*) Notice: This patent is subject to a terminal dis-  
claimer.

(21) Appl. No.: **13/299,499**

(22) Filed: **Nov. 18, 2011**

**Related U.S. Patent Documents**

Reissue of:

(64) Patent No.: **7,947,681**  
 Issued: **May 24, 2011**  
 Appl. No.: **12/577,509**  
 Filed: **Oct. 12, 2009**

U.S. Applications:

(63) Continuation of application No. 12/329,838, filed on  
Dec. 8, 2008, now Pat. No. 7,612,073, which is a  
continuation of application No. PCT/US2008/060041,  
filed on Apr. 11, 2008.

(60) Provisional application No. 60/922,821, filed on Apr.  
11, 2007, provisional application No. 61/019,753,  
filed on Jan. 8, 2008.

(51) **Int. Cl.**

**A01N 43/58** (2006.01)  
**A61K 31/50** (2006.01)  
**A01N 43/60** (2006.01)  
**A61K 31/495** (2006.01)

(52) **U.S. Cl.** ..... **514/249**

(58) **Field of Classification Search** ..... **514/249**  
 See application file for complete search history.

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*Primary Examiner* — Yong Chong

(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun  
LLP

(57) **ABSTRACT**

The present invention is directed to treatment methods of  
administering tetrahydrobiopterin, including in oral dosage  
forms, in intravenous formulations, and with food. Also dis-  
closed herein are biopterin assays for measuring the amount  
of biopterin and metabolites of biopterin in a sample.

**28 Claims, 38 Drawing Sheets**

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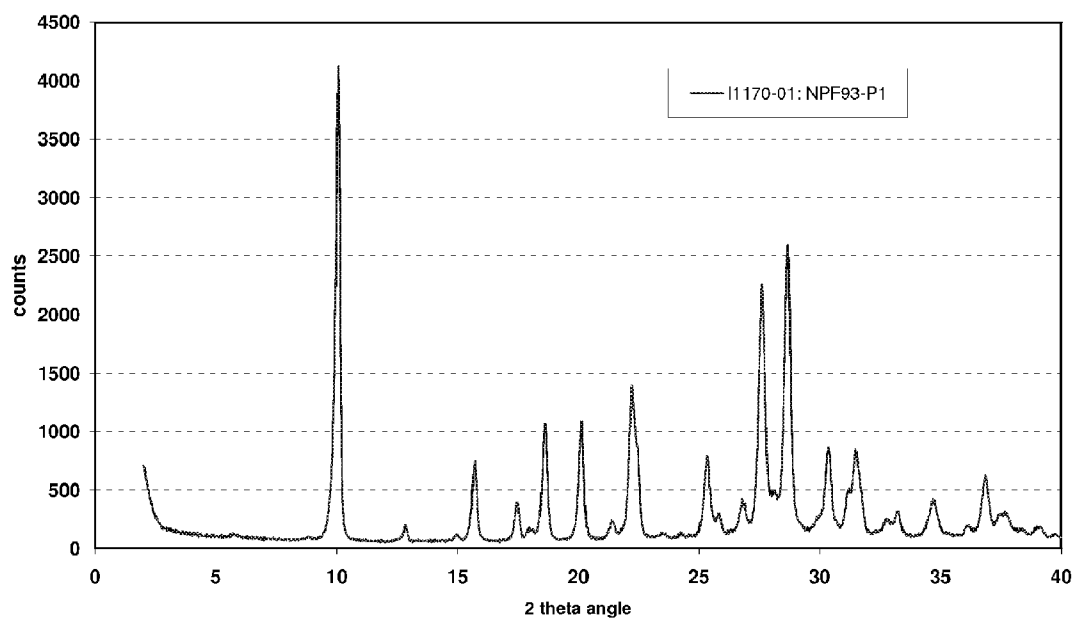
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**Figure 1**

**Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form B**



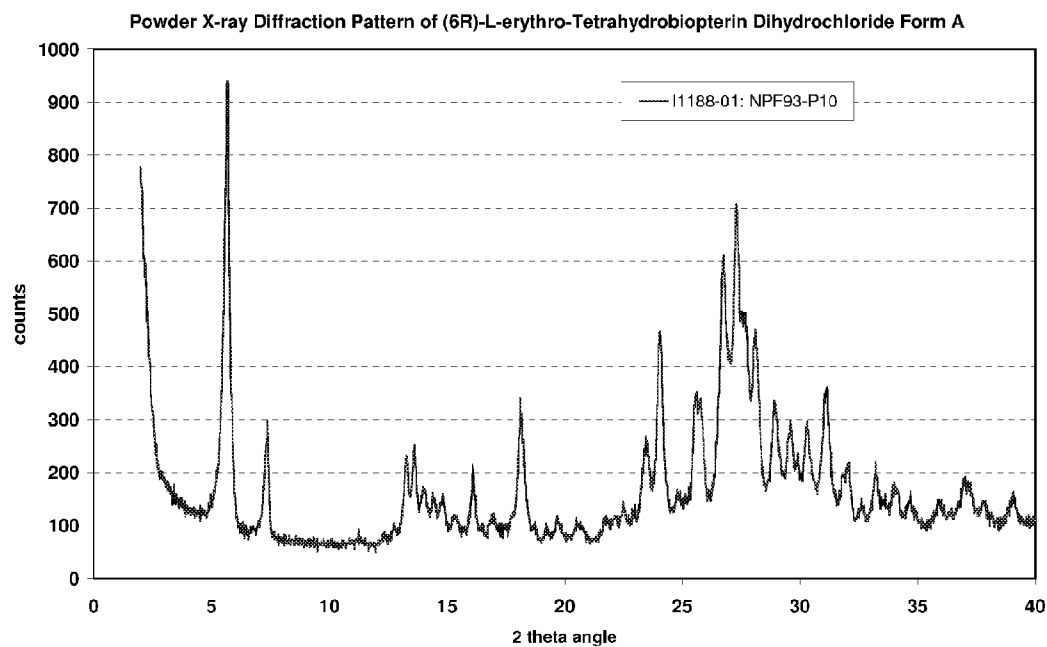
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**Figure 2**



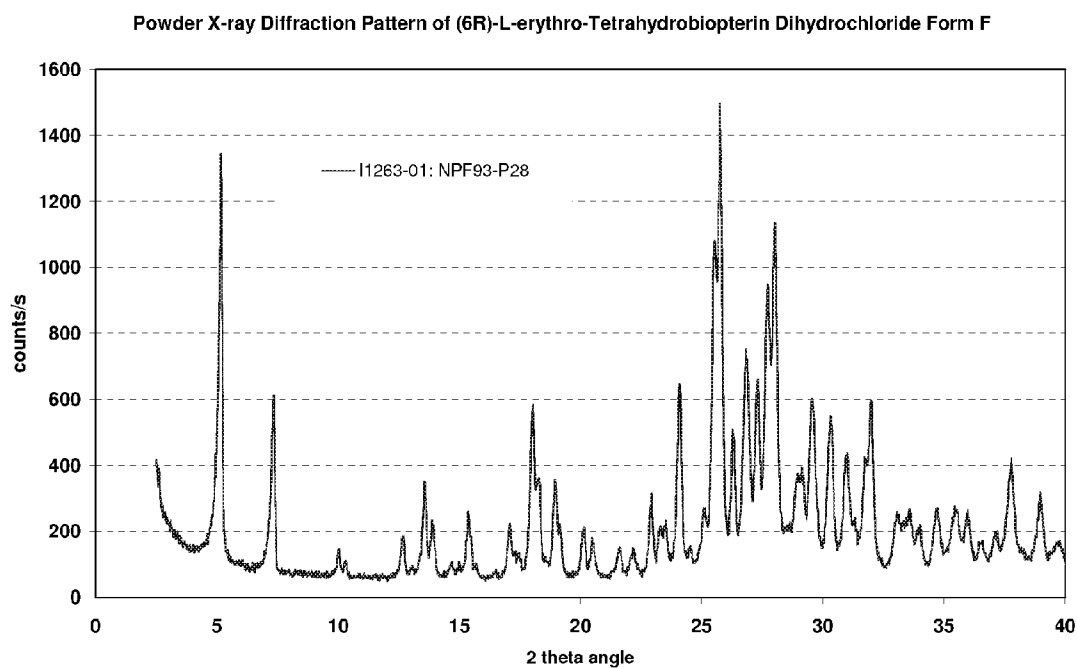
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**Figure 3**





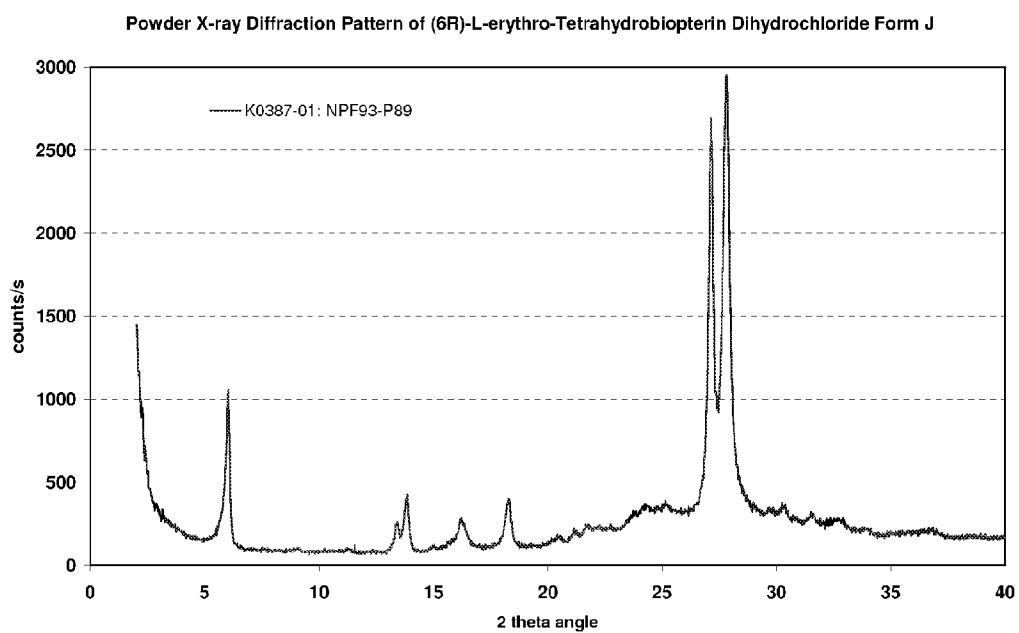
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**Figure 4**



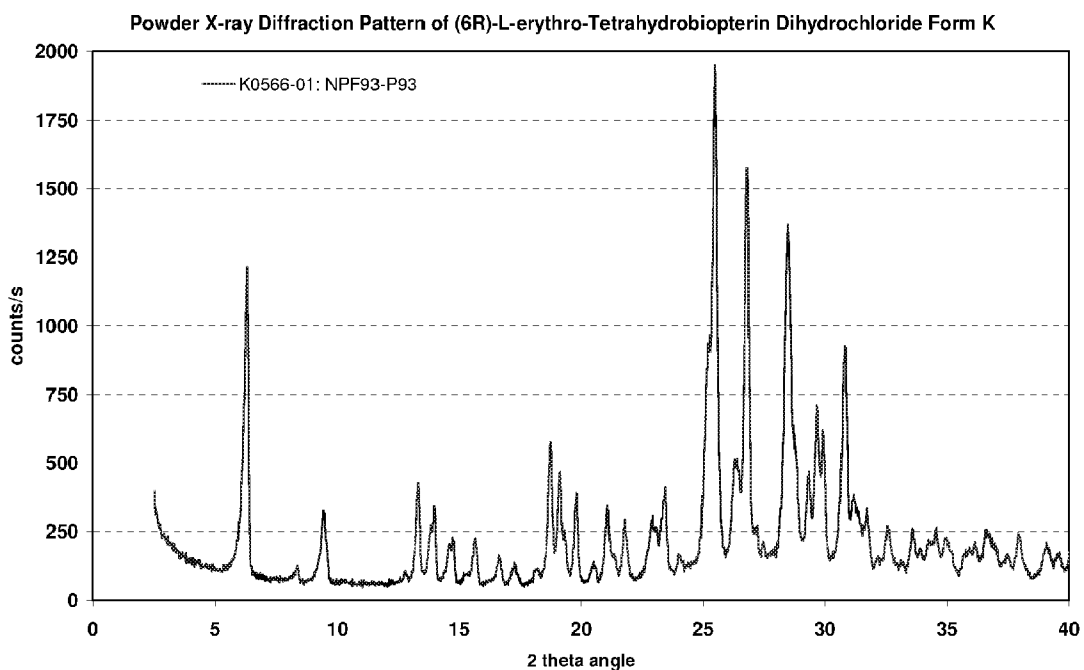
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**Figure 5**



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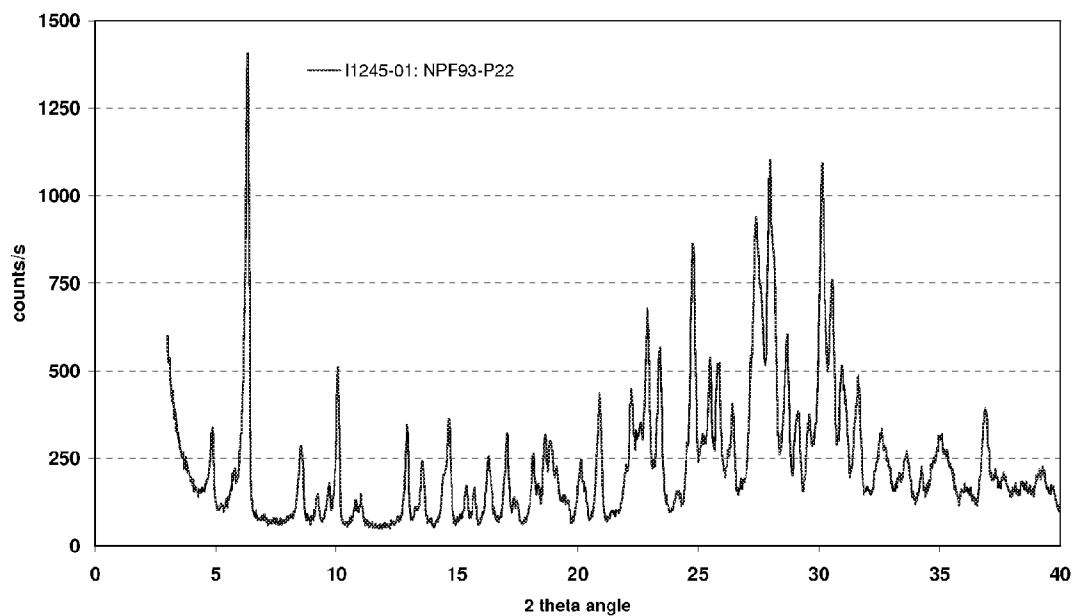
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**Figure 6**

**Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochlorid Form C**



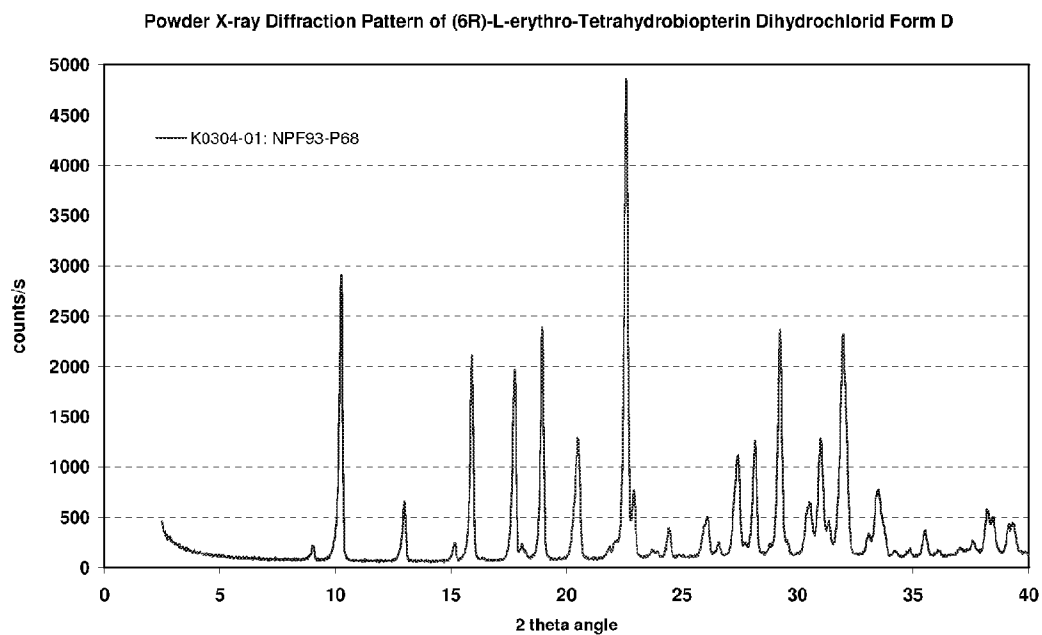
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**Figure 7**



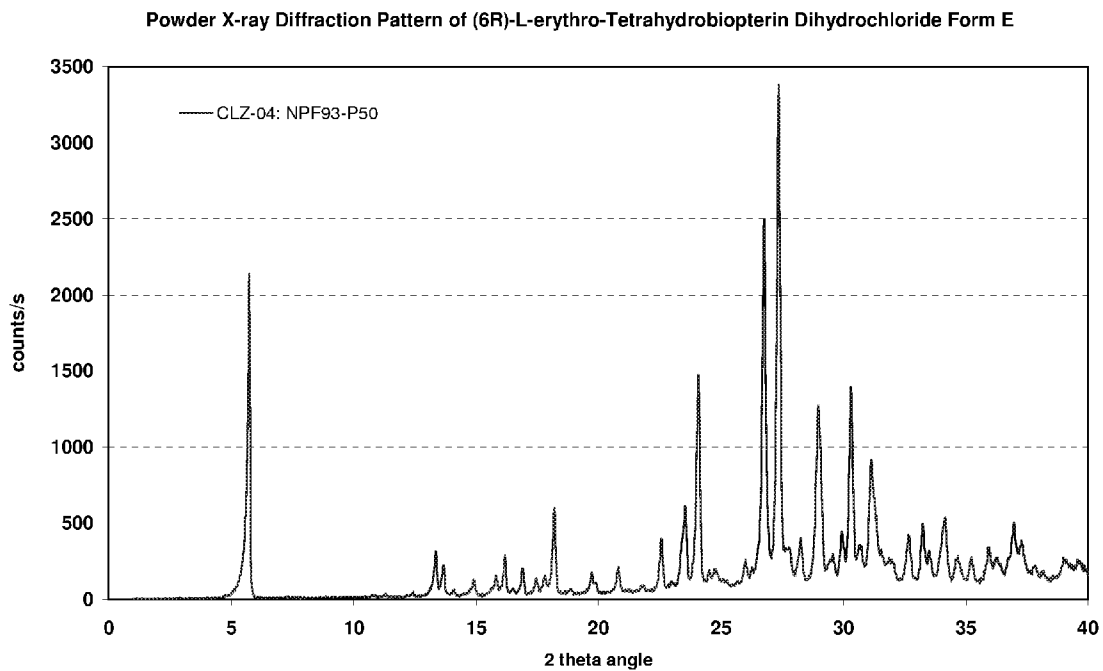
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**Figure 8**





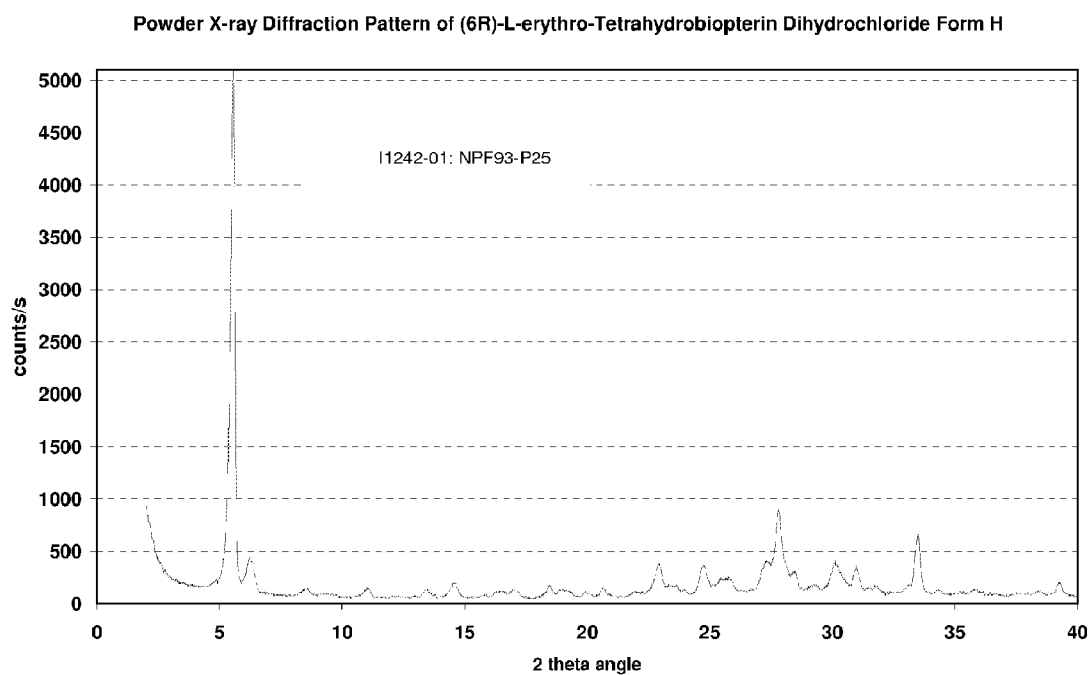
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**Figure 9**



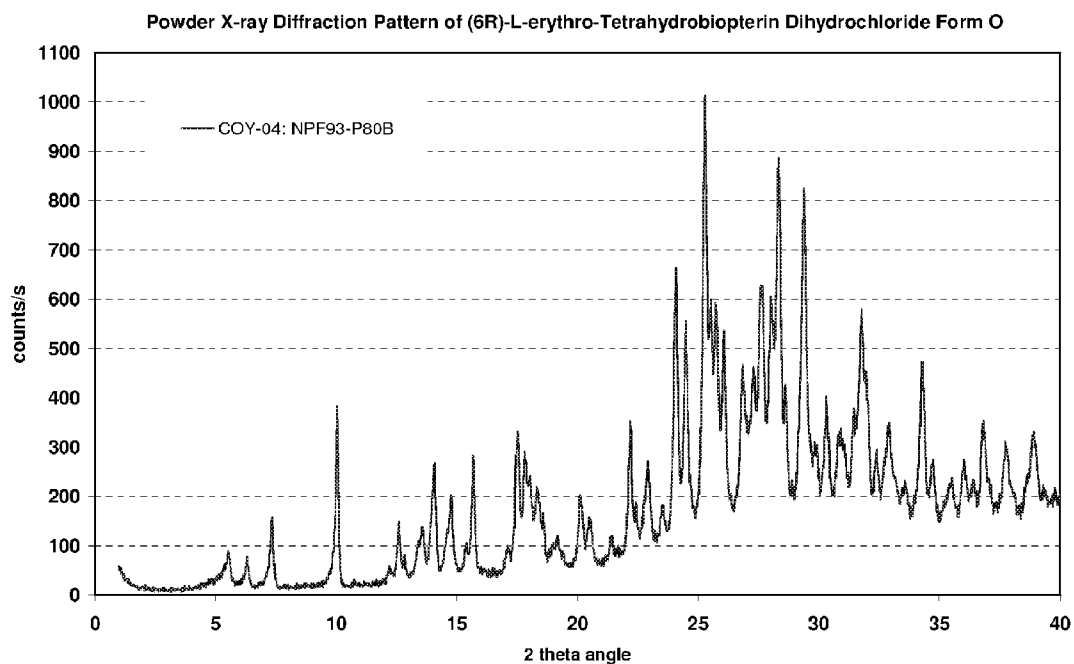
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**Figure 10**



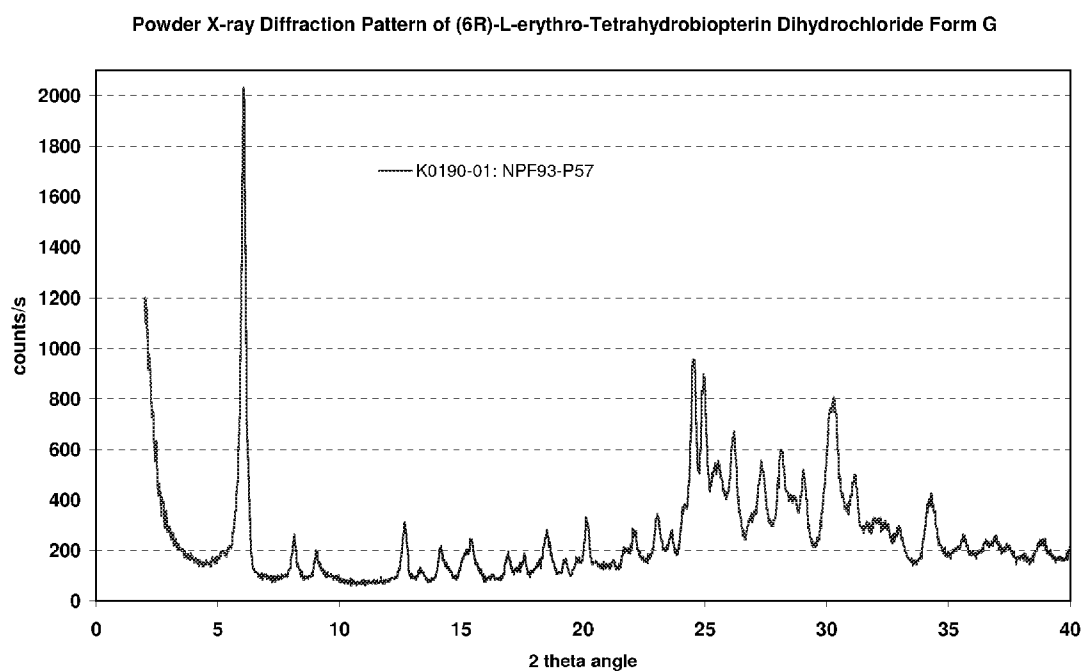
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**Figure 11**



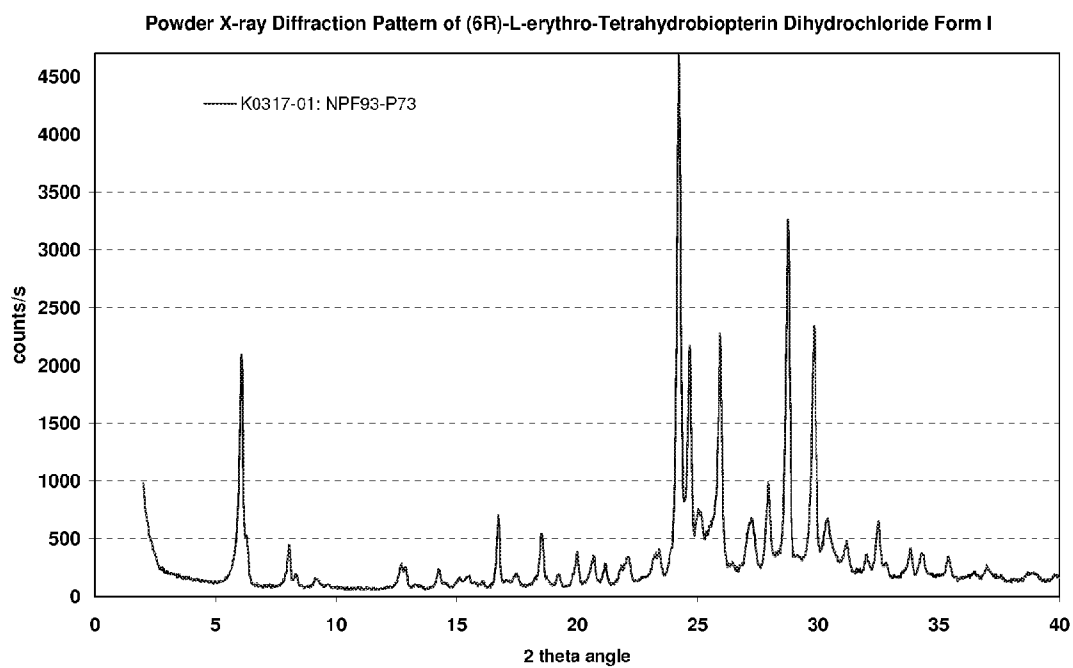
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**Figure 12**



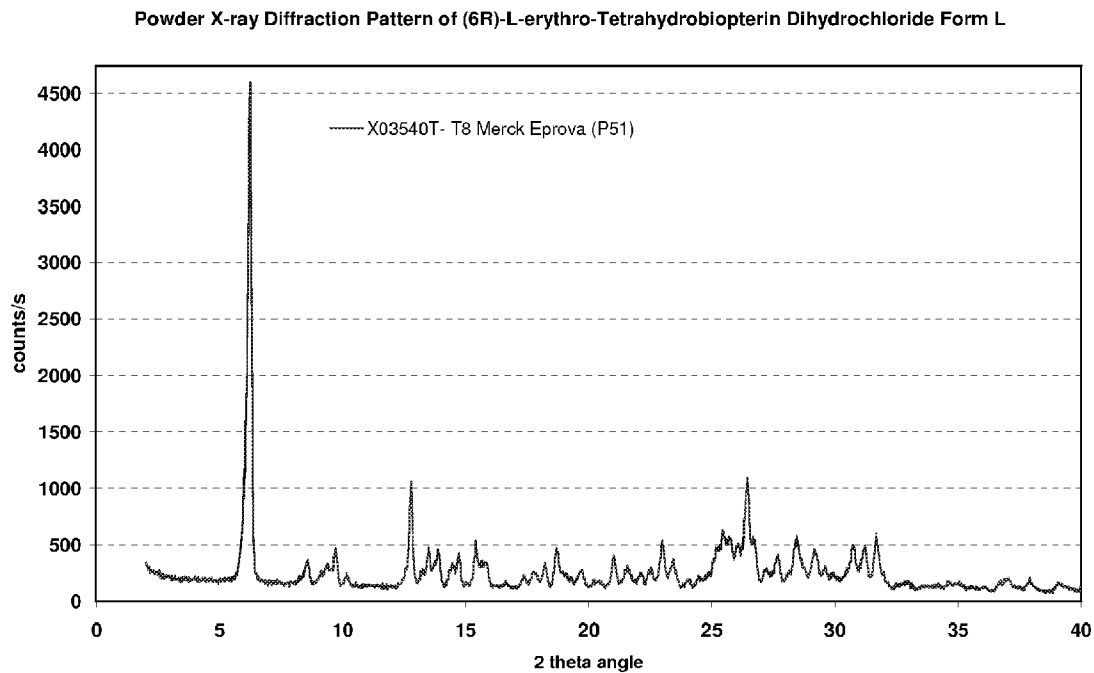
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**Figure 13**



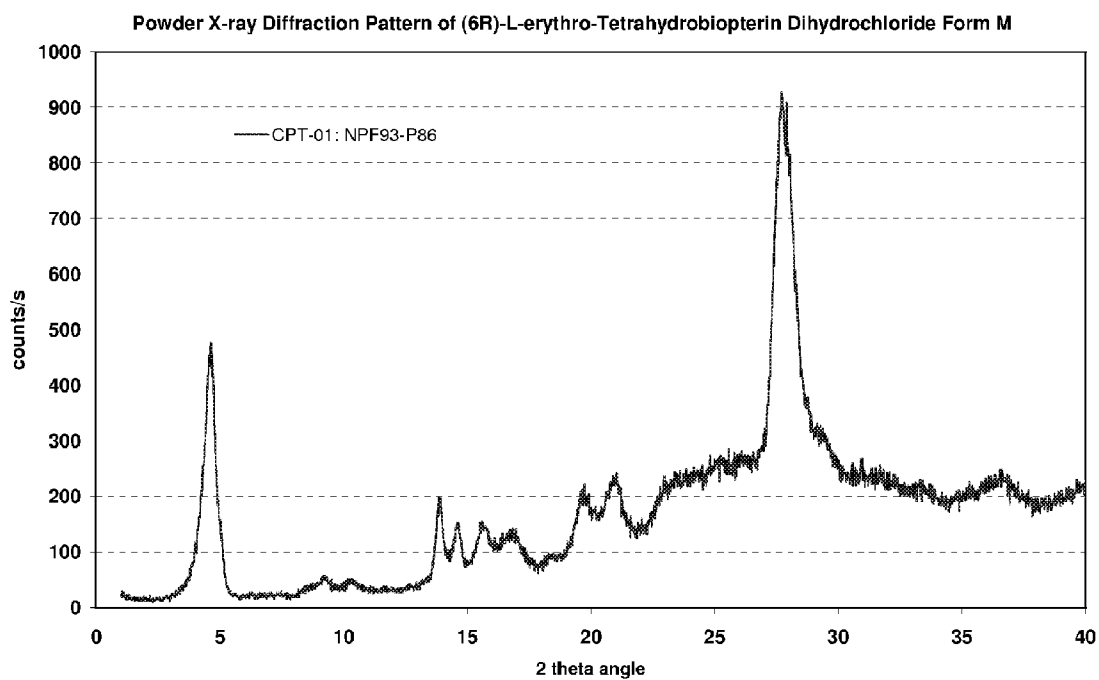
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**Figure 14**





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**Figure 15**

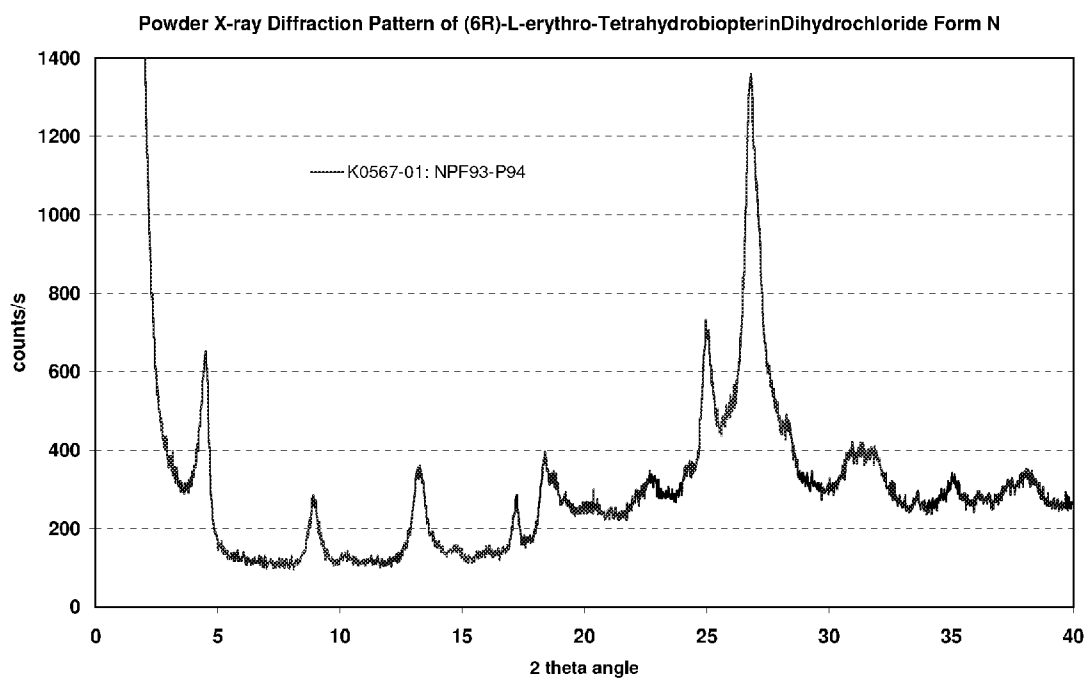
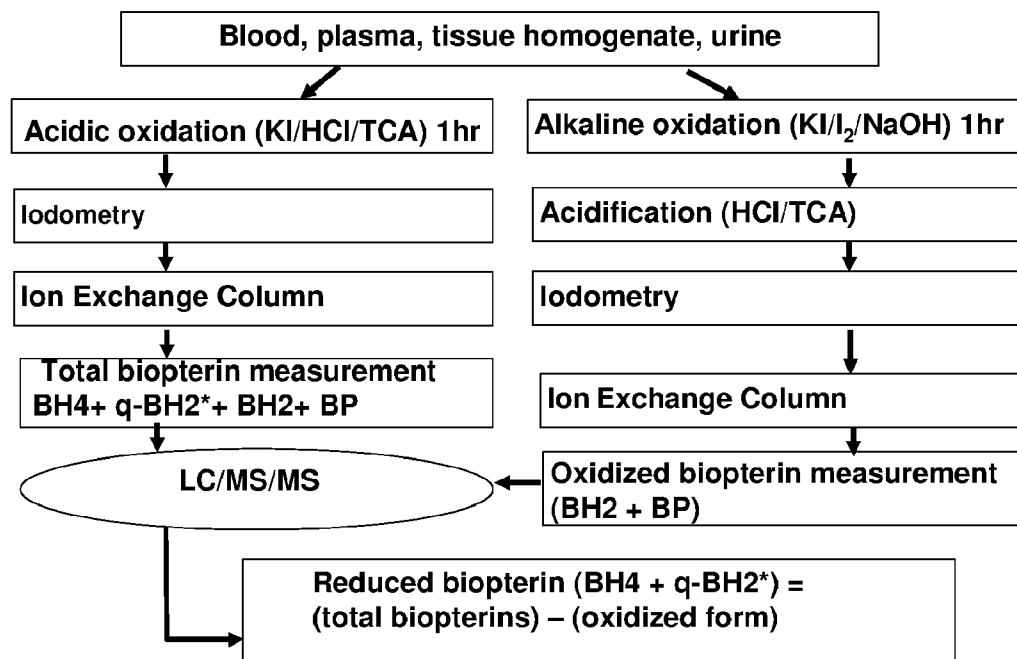


FIGURE 16 - Flow chart of bipterin measurement



\*q-BH2 is immediately reduced *in vivo* to BH4 so the measured reduced bipterin is based mainly upon BH4.

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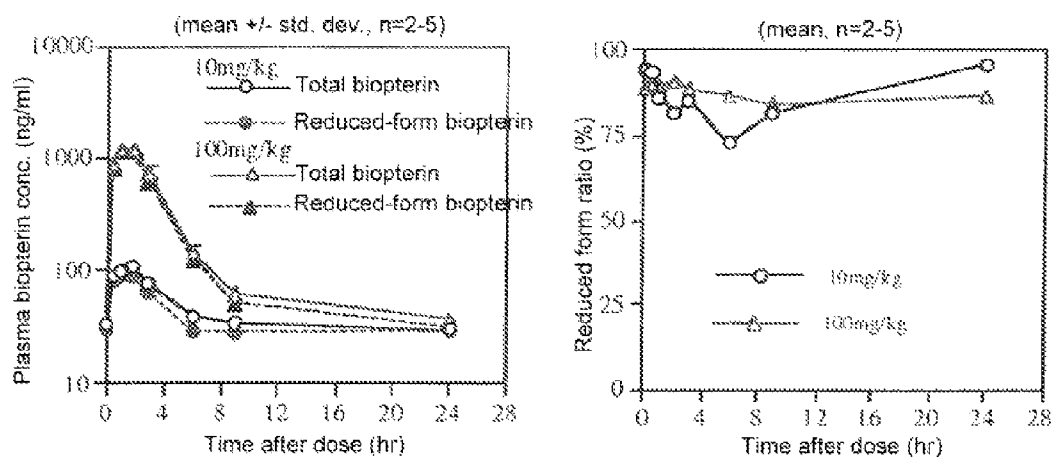
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**Figure 17**

<b>Assay</b>	<b>LC/MS/MS Determination of BH4 in human plasma</b>	<b>HPLC Determination of bioppterin in human plasma</b>
<b>Analyte Name</b>	<b>Tetrahydrobiopterin (BH4)</b>	<b>Bioppterin</b>
<b>Analyte Name (oxidation product)</b>	<b>L-biopterin</b>	<b>Bioppterin</b>
<b>Internal Standard</b>	<b>Iribesartan</b>	<b>Bioppterin</b>
<b>Analytical Method Type</b>	<b>LC/MS/MS</b>	<b>HPLC (Ex 365 nm/Em 473 nm)</b>
<b>Extraction Method</b>	<b>Protein Precipitation</b>	<b>Protein Precipitation</b>
<b>QC Concentrations</b>	<b>5, 15, 150, and 800 ng/mL BH4</b>	<b>1, 10, and 40 ng/mL bioppterin</b>
<b>Standard Curve Concentrations</b>	<b>5, 15, 50, 100, 300, 500, and 1000 ng/mL</b>	<b>0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL</b>
<b>Lower Limit of Quantitation</b>	<b>5 ng/mL</b>	<b>(5 ng/mL bioppterin)</b>
<b>Upper Limit of Quantitation</b>	<b>1000 ng/mL</b>	<b>(50 ng/mL bioppterin)</b>
<b>Average Recovery of Drug</b>	<b>65.3%</b>	<b>??</b>
<b>Average Recovery of Internal Standard</b>	<b>94%</b>	<b>74 -94%</b>
<b>QC Intraday Precision Range</b>	<b>4.7 to 14.5 %CV</b>	<b>0.8 to 1.3</b>
<b>QC Intraday Accuracy Range</b>	<b>-7.1 to 7.4 %Diff</b>	<b>2.8 to 6.1</b>
<b>QC Interday Precision Range</b>	<b>7.4 to 16.4 %CV</b>	<b>0.6 to 4.9</b>
<b>QC Interday Accuracy Range</b>	<b>-8.3 to 3.7 %Diff</b>	<b>??</b>
<b>Stock Solution Solvent</b>	<b>MeOH:DMSO/50:50 (v:v)</b>	<b>Ammonium phosphate buffer</b>
<b>Benchmark Stability in human Plasma</b>	<b>4.5 hrs at RT</b>	<b>??</b>
<b>Freeze/thaw Stability in human plasma</b>	<b>4 cycles at -70 C</b>	<b>2 cycles at -20 C</b>
<b>Conversion Ratio from BH4 to L-Biopterin</b>	<b>47% (at 12 weeks)</b>	<b>??</b>
<b>Long-term stability in K2 EDTA plasma</b>	<b>38 days at -70 C</b>	<b>?? (7 days at -20 C)</b>
<b>Dilution Integrity</b>	<b>1500 ng/mL diluted 10-times</b>	<b>??</b>
<b>Selectivity</b>	<b>BH4</b>	<b>Total bioppterin</b>

FIGURE 18

Plasma Bioppterin Concentration And Reduced-Form Ratio  
After Single-Dose Administration Of Sapropterin to Rats



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**Figure 19**  
**Pharmacokinetic Parameters of Total Biopterins in Plasma**  
**after a Single Oral Administration of Sapropterin to Rats**

<b>Dose</b>	<b>Administration</b>	<b>Cendo<sup>1</sup></b>	<b>Cmax</b>	<b>ΔCmax<sup>2</sup></b>	<b>Tmax</b>	<b>ΔAUC<sup>3</sup></b>	<b>T<sub>1/2</sub></b>	<b>F<sup>4</sup></b>
<b>(mg/kg)</b>	<b>Route</b>	<b>(ng/ml)</b>	<b>(ng/ml)</b>	<b>(ng/ml)</b>	<b>(hr)</b>	<b>(ng-hr/ml)</b>	<b>(h)</b>	<b>(%)</b>
100	p.o.	38.2	1227	1189	1.0	4571	1.1(2-6 h)	11.8
10	p.o.	33.5	108	75	2.0	265	1.1(2-6h)	6.8
10	i.v.	33.5	—	—	—	3881	0.6(0.5–3h)	
1	i.v.	33.5	—	—	—	529	0.3(0.5–3h)	

<sup>1</sup> Endogenous total biopterin concentration

<sup>2</sup> Cmax – Cendo

<sup>3</sup> Computed based on trapezoidal rule, by using the value (ΔC) obtained by subtracting Cendo from the actually measured value (C) of plasma concentration.

<sup>4</sup> Bioavailability (F) was computed by using ΔAUC at the time of 10-mg/kg intravenous injection:

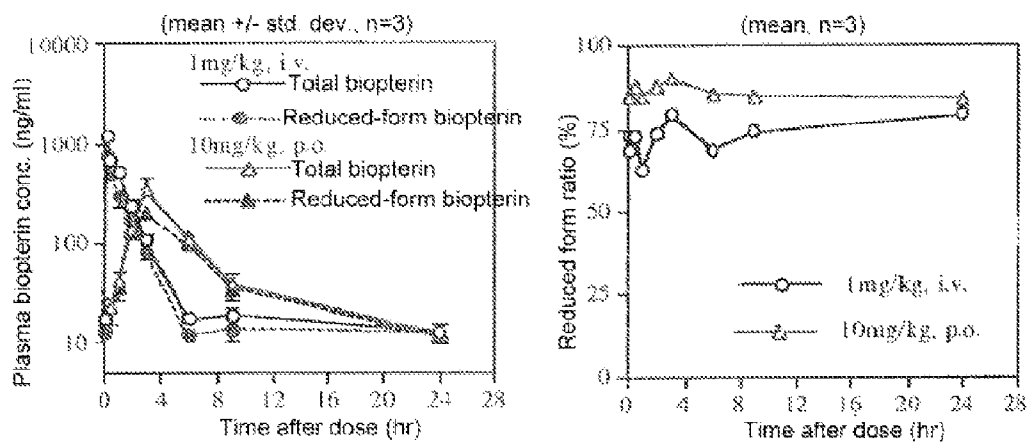
$$F = [\Delta AUC_{po}] / [DOSL_{po}] / [\Delta AUC_{iv}] / 10 \times 100 (\%)$$

Sapropterin Lot #s: 8886202, 8885Y05

(mean values of 2 to 5 animals)

FIGURE 20

**Plasma Biopterin Concentration and Reduced-Form Ratio  
After a Single-Dose Administration of Sapropterin in Monkeys**





**U.S. Patent****Nov. 6, 2012****Sheet 21 of 38****US RE43,797 E****FIGURE 21**

**Pharmacodynamic Parameters of Total Biopterins in Plasma  
after Single-Dose Administration of Sapropterin to Monkeys**

<b>Dose</b> <b>(mg/kg)</b>	<b>Administration</b> <b>Route</b>	<b>Cendo<sup>1</sup></b> <b>(ng/ml)</b>	<b>Cmax</b> <b>(ng/ml)</b>	<b>ΔCmax<sup>2</sup></b> <b>(ng/ml)</b>	<b>Tmax</b> <b>(hr)</b>	<b>ΔAUC<sup>3</sup></b> <b>(ng-hr/ml)</b>	<b>T<sub>1/2</sub></b> <b>(hr)</b>	<b>F<sup>4</sup></b> <b>(%)</b>
10	p.o.	17.4±1.3	344±149	344±148.5	2.9±0.2	1301±144	1.42±0.17	9.0
1	i.v.	17.1±2.1				1449±68.4	0.82±0.14	

<sup>1</sup> Endogenous total biopterin concentration<sup>2</sup> Cmax – Cendo<sup>3</sup> Computed based on trapezoid rule, by using the value (ΔC) obtained by subtracting Cendo from the actually measured value (C) of plasma concentration.<sup>4</sup> Bioavailability (F) was computed by using ΔAUC at the time of 1-mg/kg intravenous injection.
$$F = [\Delta AUC_{po}] / [DOSI_{po}] / [\Delta AUC_{iv}] / 1 \times 100 (\%)$$

Sapropterin Lot #s:8886202, 8885Y05

(mean value ± standard error, n = 3)

Figure 22: Schedule of Events

Visit	Open-Label Treatment					Follow-up	
	Screening	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	
Informed consent	X						
Weight	X	X <sup>5</sup>				X	
Vital signs	X	X	X	X	X	X	
Physical examination	X					X	
Clinical laboratory tests <sup>1</sup>	X					X	
Pregnancy test <sup>4</sup>	X	X			X	X	
Urine Drugs of Abuse Screen <sup>2</sup>	X	X	X	X	X	X	
Concomitant medications	X	X	X	X	X	X	
Adverse events		X	X	X	X	X	
Blood PK sampling <sup>3</sup>		X	X	X	X	X	
Dispense study drug		X	X	X	X	X	

<sup>1</sup> Clinical laboratory tests included hematology, chemistry, urinalysis, Hepatitis B & C, and HIV at screening. Hematology, chemistry and urinalysis only were repeated at study discharge. Approximately 20 mL of blood was collected at visits that included clinical laboratory tests.

<sup>2</sup> Urine drugs screen to include testing for amphetamines, benzodiazepines, barbiturates, cocaine, cannabinoids, and opiates.

<sup>3</sup> Pharmacokinetic samples were taken at the following timepoints during each treatment period: pre-dose, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post dose. Approximately 80 mL of blood was drawn during each treatment period (5 mL per timepoint) for the PK analysis.

<sup>4</sup> Additional urine pregnancy tests were performed at any visit at which pregnancy status was in question, and serum pregnancy tests were performed in the event of any positive or equivocal urine pregnancy test results.

<sup>5</sup> Weight at the Week 1 treatment period was used to calculate dose.

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FIGURE 23

**Mean Plasma Concentrations of BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers – Linear Axes**

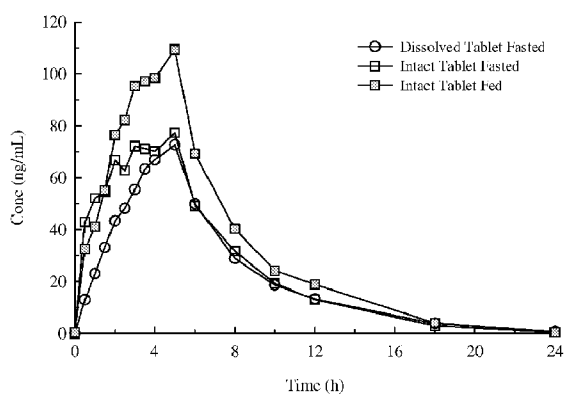


FIGURE 24

Mean Plasma Concentrations of BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers – Semi-Logarithmic Axes

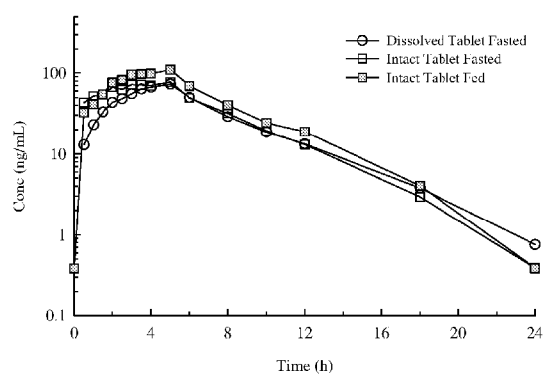


FIGURE 25

**Summary of Pharmacokinetic Parameters for BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers**

Parameter <sup>1</sup>	Dissolved Tablet Fasted	Intact Tablet Fasted	Intact Tablet Fed
C <sub>max</sub> (ng/mL)	80.3 ± 63.3 [30] (69.4)	91.2 ± 36.3 [30] (84.0)	121 ± 33.6 [30] (116)
T <sub>max</sub> (h)	4.00 [30] (2 - 6)	3.50 [30] (1 - 5)	4.00 [30] (1 - 5)
AUC(0-t) (h•ng/mL)	479 ± 292 [30] (420)	550 ± 214 [30] (505)	709 ± 221 [30] (675)
AUC(inf) (h•ng/mL)	597 ± 336 [22] (528)	704 ± 202 [19] (670)	825 ± 256 [23] (784)
λ <sub>z</sub> (h <sup>-1</sup> )	0.2101 ± 0.1326 [22]	0.2099 ± 0.0942 [19]	0.2104 ± 0.0918 [23]
t <sub>1/2</sub> (h)	5.31 ± 4.42 [22]	4.47 ± 3.37 [19]	4.28 ± 2.79 [23]

<sup>1</sup>Mean ± standard deviation except for T<sub>max</sub> for which the median is reported. Numbers in square brackets are the number of subjects for which the parameter could be estimated and numbers in parentheses are the geometric means for C<sub>max</sub>, AUC(0-t), and AUC(inf) and the ranges for T<sub>max</sub>.

FIGURE 26

**Statistical Comparison of Pharmacokinetic Parameters for BH<sub>4</sub> after Oral Administration of 10 mg/kg of Phenoptin as Dissolved and Intact Tablets under Fasted Conditions and Intact Tablets under Fed Conditions to Healthy Volunteers**

Parameter	Geometric Mean Ratio (%) <sup>1</sup>			
	Estimate	90% Confidence Interval		
Intact vs. Dissolved Tablet (Fasted)				
C <sub>max</sub>	120.98	104.21	→	140.44
AUC(0-t)	120.33	104.12	→	139.06
AUC(inf)	118.04	98.16	→	141.96
Intact Tablet — Fed vs. Fasted				
C <sub>max</sub>	138.63	119.42	→	160.93
AUC(0-t)	133.69	115.68	→	154.50
AUC(inf)	125.61	104.29	→	151.30

<sup>1</sup>Based on analysis of natural log-transformed data.



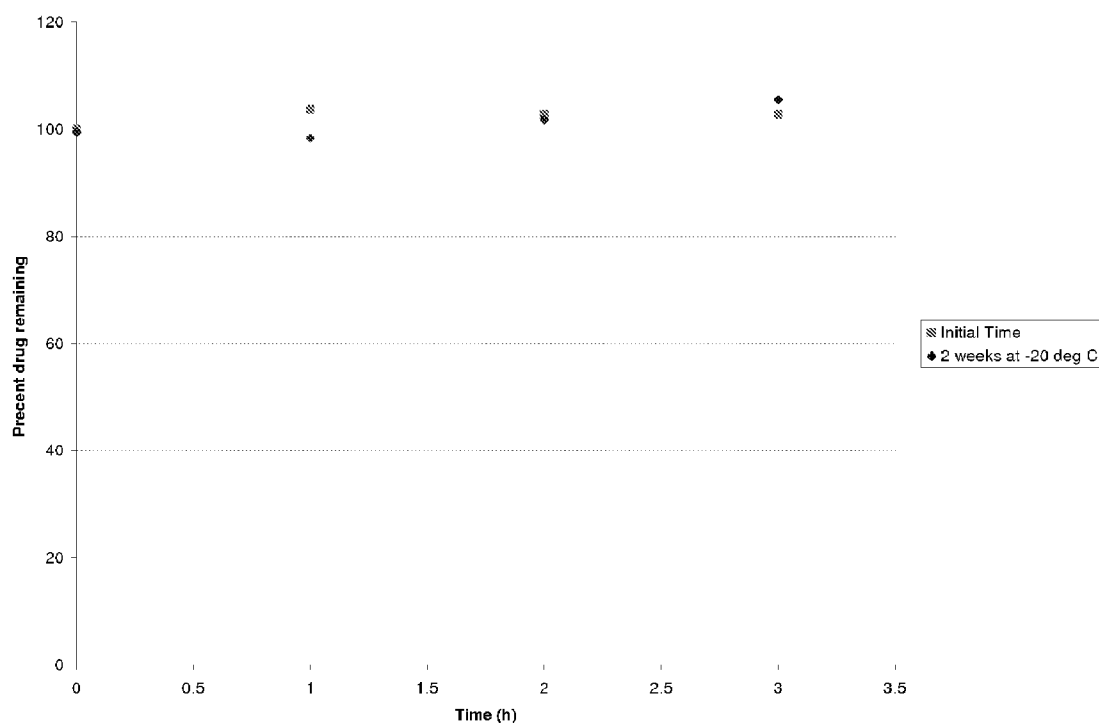
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**US RE43,797 E****FIGURE 27**

Stability of BH4 (1 mg/mL) in 5% mannitol aqueous solution



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FIGURE 28

Dissolution Profile GMO Capsule Prototype

USP Paddle Apparatus, 50 rpm, 37°C, 900 mL 0.1 N HCl (110210-28, 110210-76E)

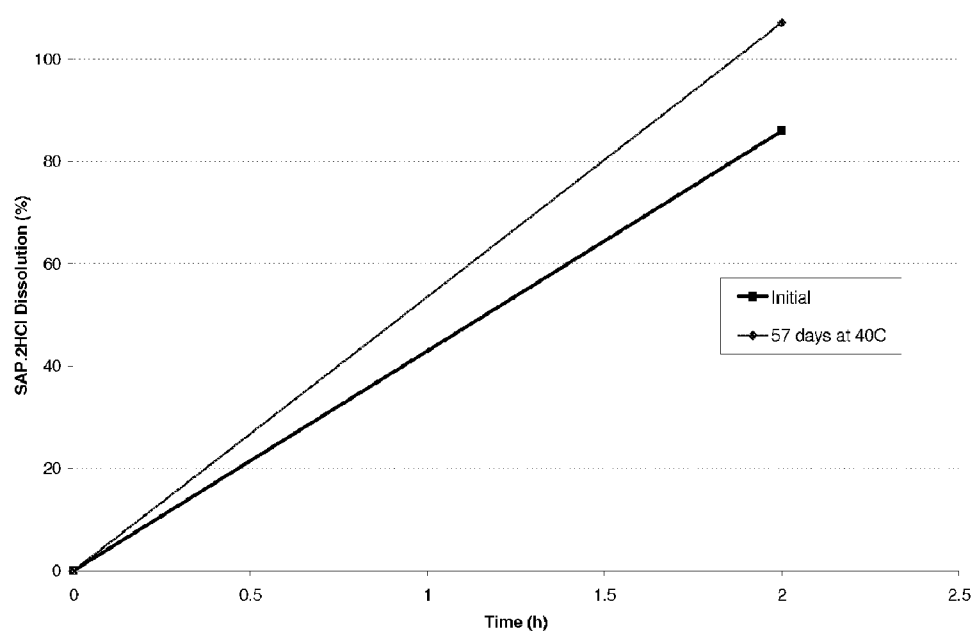


FIGURE 29

Dissolution Profile of Bioadhesive Tablet Prototype (80 mg BH4)

USP Paddle Apparatus, 50 rpm 37°C, 900 mL 0.1N HCl (11229-04, 11229-85)

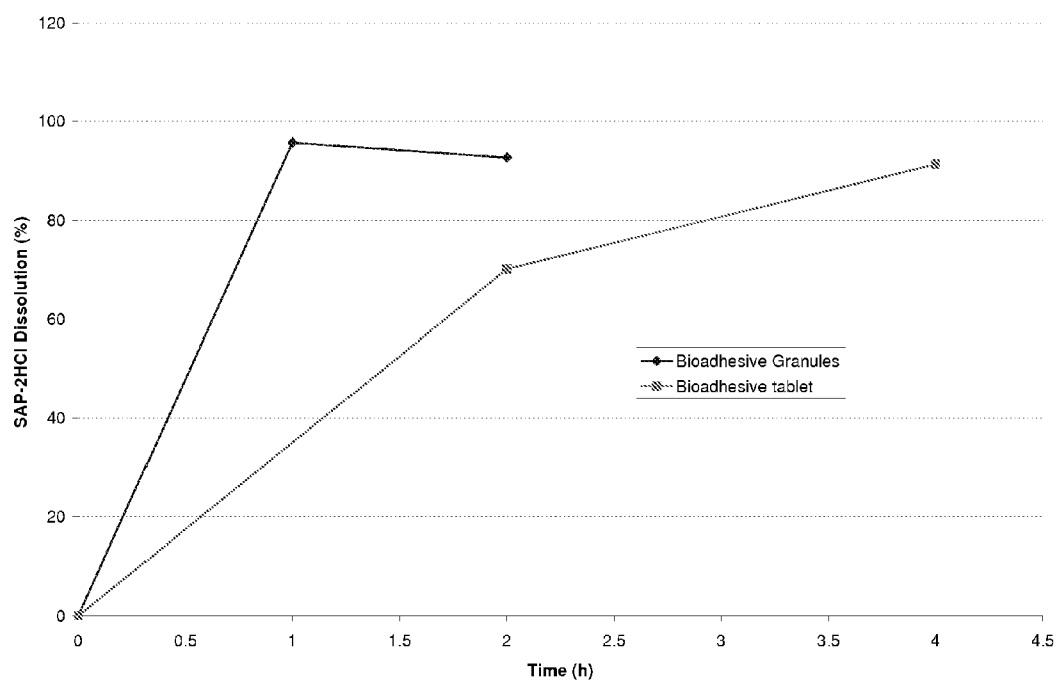
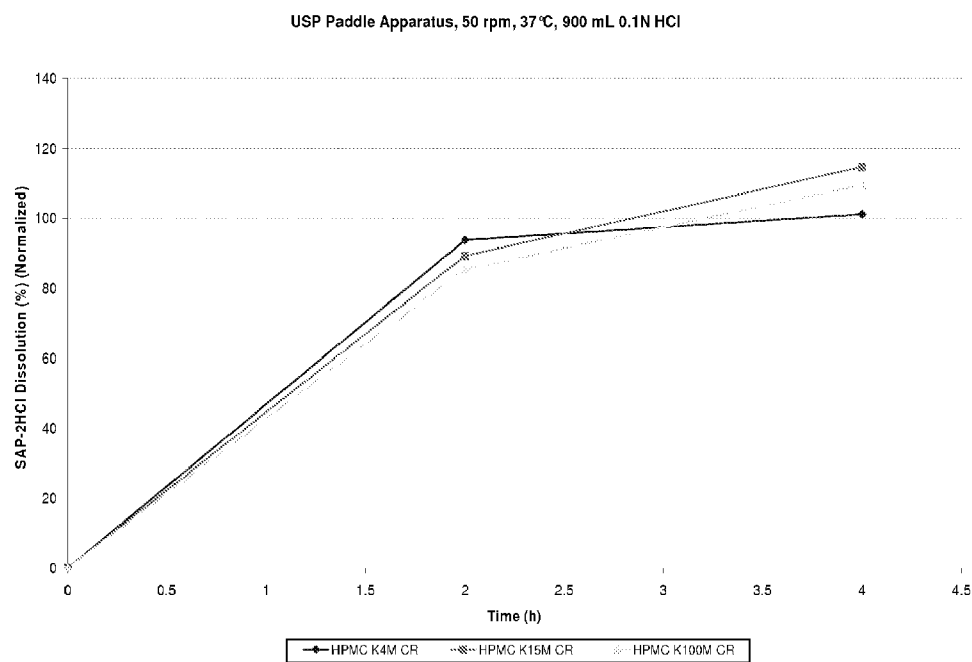


FIGURE 30

Dissolution Profile of Sustained Release Prototype (80 mg SAP-2HCl) with 20% HPMC of various viscosity grade



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Dissolution Profile of Sustained Release Prototypes (80 mg BH4) containing 20% to 40% Methocel K100M CR

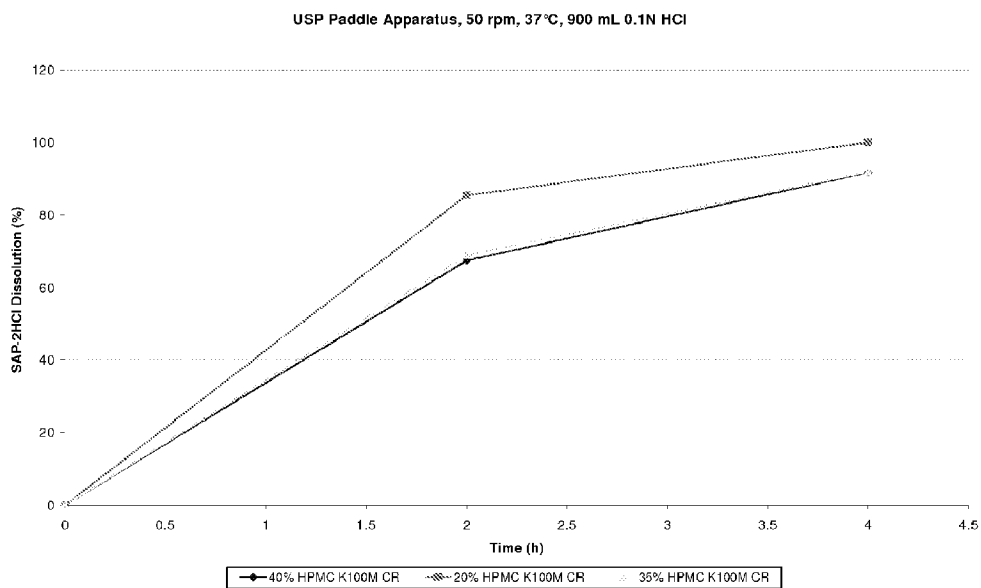


FIGURE 32

Schematic Diagram of Floating Dosage Form

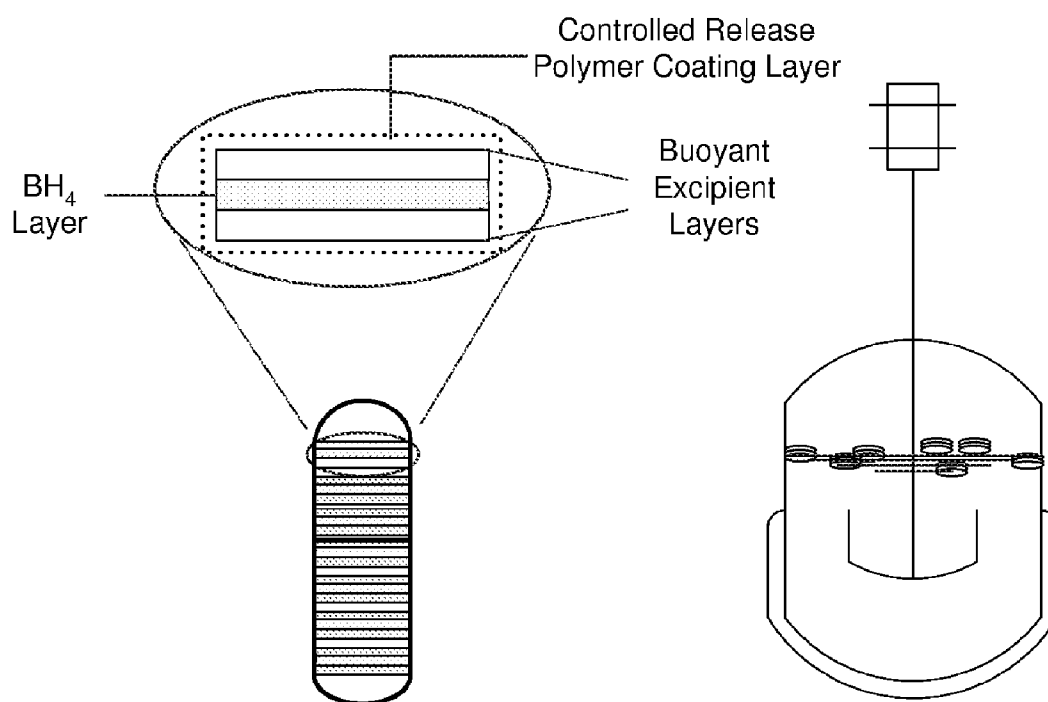




FIGURE 33

Dissolution Profile of Floating System Prototype (80 mg BH4) with Varying Levels of PEG Coating

USP Paddle Apparatus, 50 rpm 37°C, 900 mL 0.1N HCl

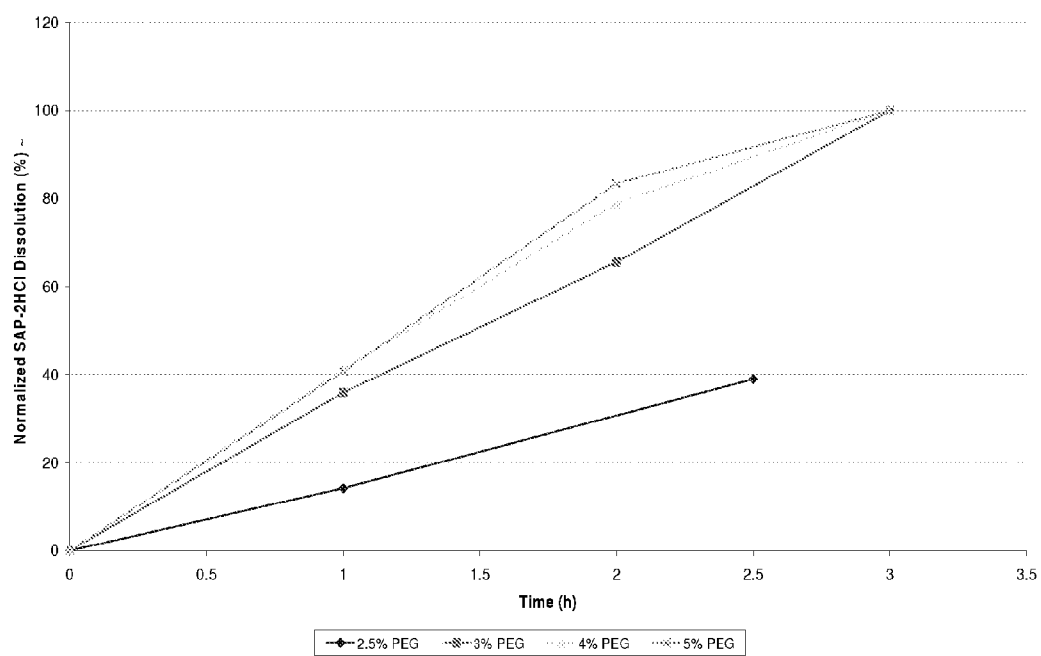
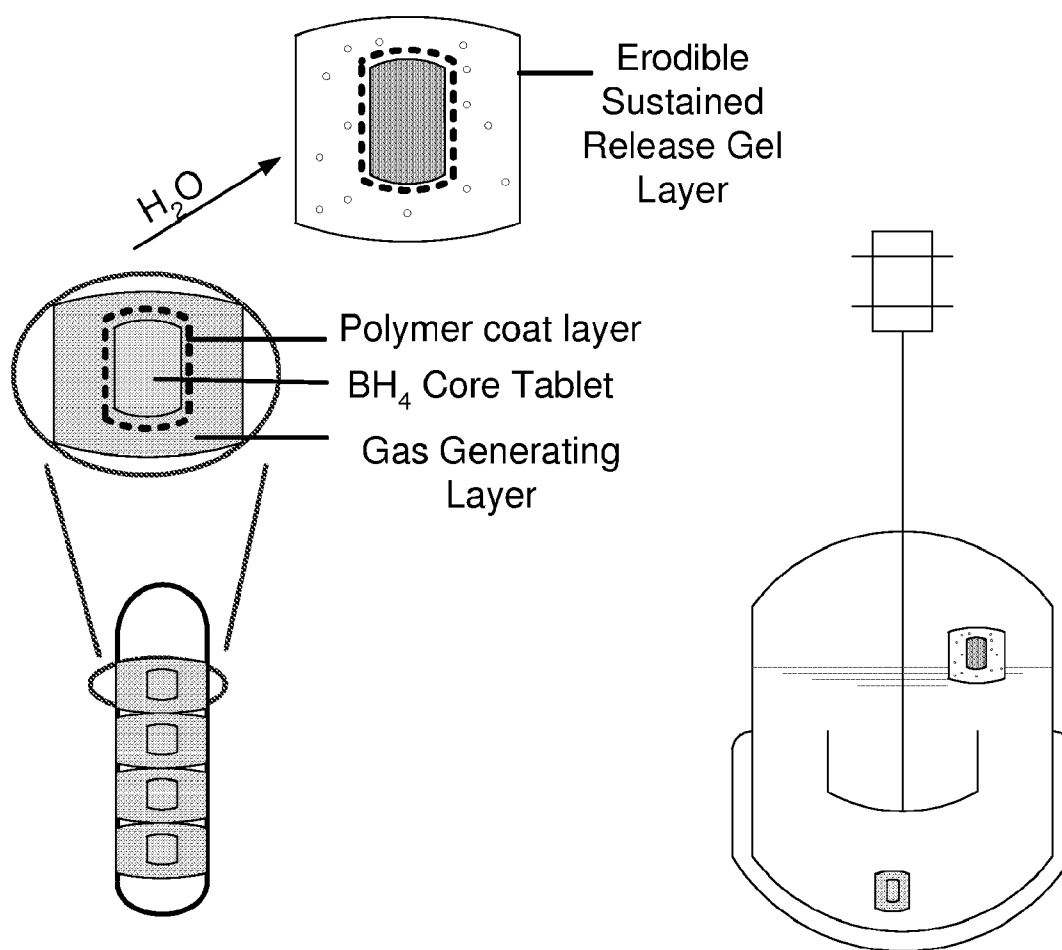


FIGURE 34

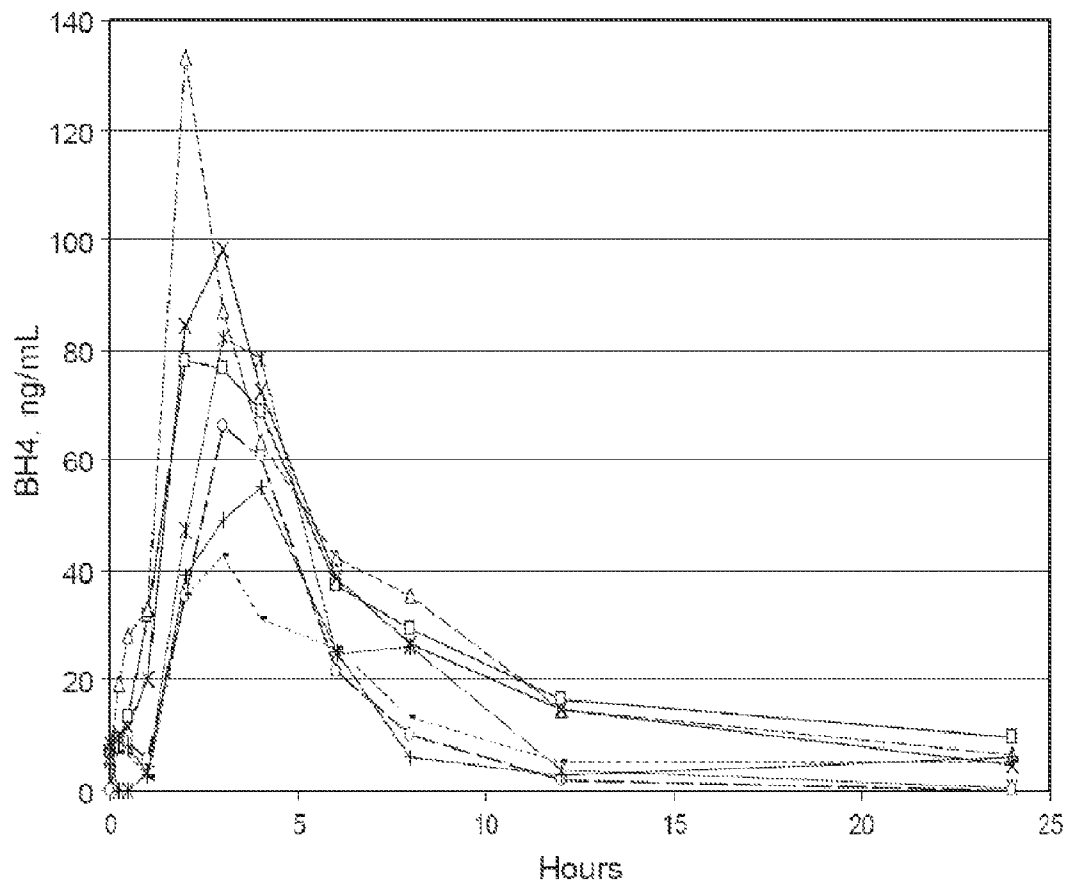
Schematic Diagram of Gas Generating Dosage Form



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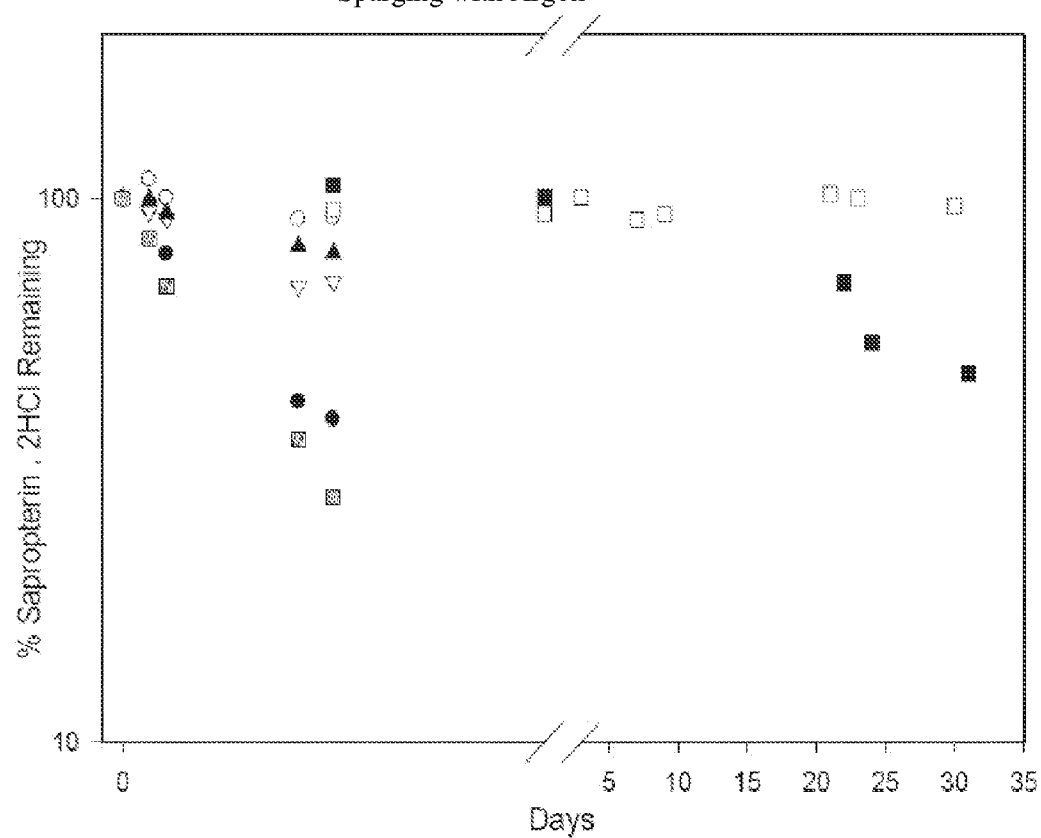
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**US RE43,797 E****FIGURE 35**

- |                                     |                                   |
|-------------------------------------|-----------------------------------|
| ○ Phase 2, dissolved Kuvan tablet   | △ Phase 3, glyceryl mon-oleate    |
| × Phase 4, bioadhesive polymer      | ★ Phase 5, sustained release form |
| ◇ Phase 6, proton donor polymer     | ⊕ Phase 7, floating dosage forms  |
| ⋯ Phase 8, bioadhesive granulations |                                   |

FIGURE 36

Stability of BH<sub>4</sub> in pH 4 Buffer in the Presence and Absence of Antioxidants and Sparging with Argon



- Buffer Only
- Buffer + Ascorbic Acid
- ▽ Buffer + Cysteine
- ▲ Sparged Argon
- Sparged Oxygen
- Buffer + Ascorbic Acid + L- Cysteine
- Buffer + Ascorbic Acid + L-Cysteine + Argon Spa

FIGURE 37

Stability of BH4 in pH 7 Buffer in the Presence and Absence of Antioxidants and  
Sparging with Argon

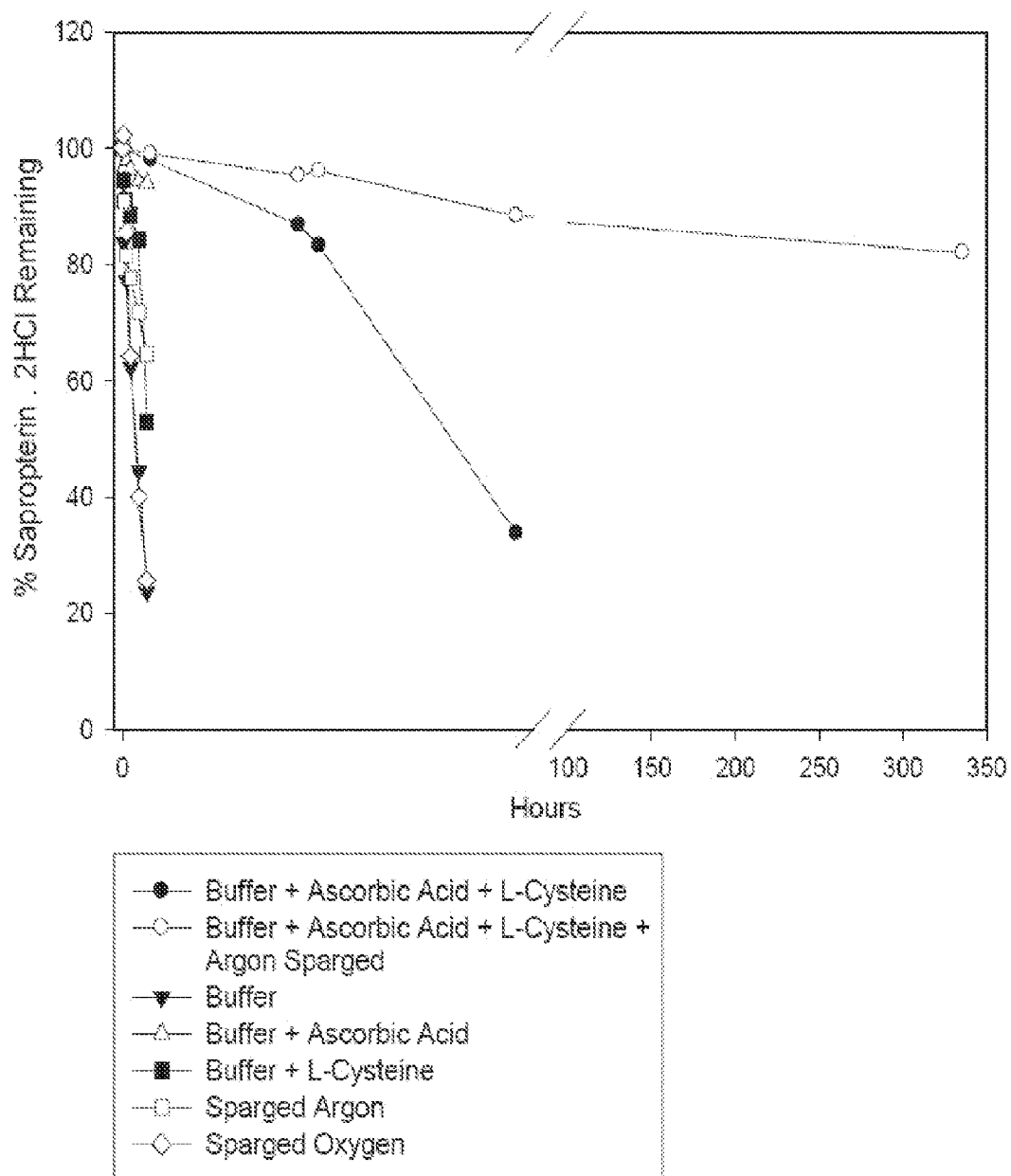
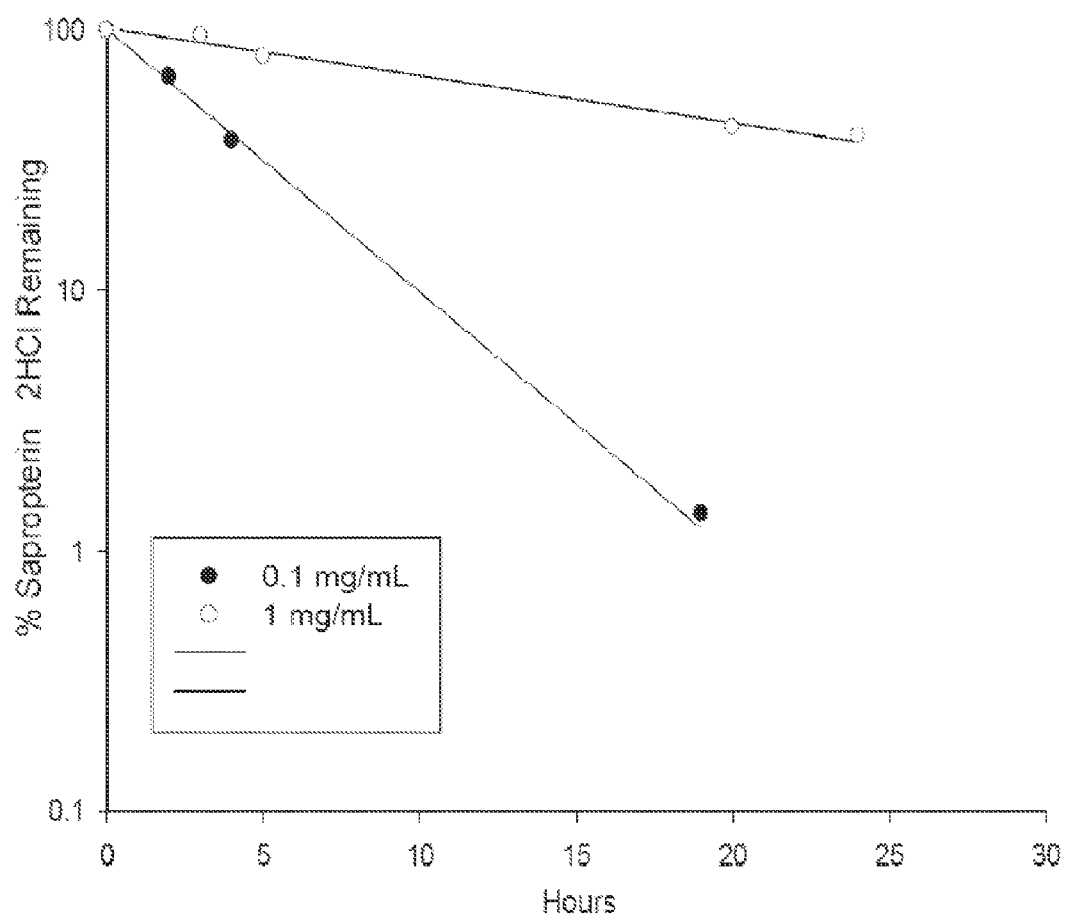


FIGURE 38

Stability of BH<sub>4</sub> Remaining in pH 4 Buffer Solution with Time as a Function of Concentration





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## METHODS OF ADMINISTERING TETRAHYDROBIOPTERIN

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.**

### CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 12/329,828 filed Dec. 8, 2008, which in turn is a continuation of International Application No. PCT/US08/060,041, filed Apr. 11, 2008, which claims priority to U.S. Provisional Application Nos. 60/922,821, filed Apr. 11, 2007, and 61/019,753, filed Jan. 8, 2008, the disclosures of which are incorporated herein by reference in their entirety.

### BACKGROUND

#### 1. Field

The present invention is generally directed to compositions and methods for treating BH4-responsive disorders, and methods and compositions for detecting and quantitating bipterins.

#### 2. Background of the Related Technology

Tetrahydrobiopterin (referred to herein as BH4) is a biogenic amine of the naturally-occurring pterin family that is a cofactor for a number of different enzymes, including phenylalanine hydroxylase (PAH), tyrosine hydroxylase, tryptophan hydroxylase and nitric oxide synthase. Pterins are present in physiological fluids and tissues in reduced and oxidized forms, however, only the 5,6,7,8, tetrahydrobiopterin is biologically active. It is a chiral molecule and the 6R enantiomer of the cofactor is known to be the biologically active enantiomer. For a detailed review of the synthesis and disorders of BH4 see Blau et al., 2001 (Disorders of tetrahydrobiopterin and related biogenic amines. In: Scriver C R, Beaudet A L, Sly W S, Valle D, Childs B, Vogelstein B, eds. The Metabolic and Molecular Bases of Inherited Disease. 8th ed. New York: McGraw-Hill, 2001: 1275-1776).

Fiege, et al., Molecular Genetics and Metabolism 81:45-51 (2004) studied pharmacokinetics of orally administered tetrahydrobiopterin (BH4) and suggested a "rather large variability of orally administered BH4, probably due to different absorption in the gut and/or to the first passage effect."

Use of tetrahydrobiopterin has been proposed for treating a variety of different disease states, and there exists a need for alternative and improved methods of administering this drug.

### SUMMARY OF THE INVENTION

The present invention relates to methods of administering 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4), or a pharmaceutically acceptable salt thereof, in a manner that improves or maximizes its oral bioavailability and/or improves or optimizes the consistency of oral bioavailability from one administration to the next. Such methods can be applied in the treatment of any BH4-responsive disorder, including metabolic diseases, cardiovascular diseases, anemia, and neuropsychiatric disorders. The methods of the invention advantageously allow better control of clinical symptoms, e.g. reduced fluctuation in plasma phenylalanine levels, blood pressure, neurotransmitter levels, or other clinical parameters.

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As used herein, BH4 refers to 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin. The term BH4 as used herein is also to be understood to optionally mean a pharmaceutically acceptable salt of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin, unless the context dictates otherwise.

In a first aspect, the invention provides methods of orally administering to a patient in need thereof a purified preparation of BH4.

In an exemplary embodiment, the methods comprise the step of informing the patient that absorption of tetrahydrobiopterin is increased when it is ingested with food compared to when ingested without food. In some embodiments, the patient is informed that ingestion shortly following a meal, for example, a high-fat, high-calorie meal, results in an increase in any one, two, three or all of the following parameters: mean plasma concentration, Cmax, AUC, AUC(0-t) and/or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 with a high-fat meal increases Cmax and AUC compared to administration of BH4 without food (in a fasting condition). In some embodiments, the relative increase can be at least 20% or 30% or more.

In alternative embodiments or in addition to the preceding embodiments, the method of administering tetrahydrobiopterin comprises informing the patient that absorption of tetrahydrobiopterin is increased when ingested as an intact tablet compared to when ingested after being dissolved in liquid. In some embodiments, the patient is informed that ingestion of intact tablets results in an increase in any of the following parameters: mean plasma concentration, Cmax, AUC, AUC(0-t) or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 as an intact tablet increases Cmax and AUC compared to administration of BH4 after being dissolved in a liquid. In some embodiments, the relative increase can be at least 20% or more.

Any of the preceding methods may be carried out by providing or administering tetrahydrobiopterin in a container containing printed labeling informing the patient of the change in absorption parameters described above.

Optionally, the methods of the invention also comprise the step of providing to the patient in need thereof a therapeutically effective amount of tetrahydrobiopterin. The therapeutically effective amount will vary depending on the condition to be treated, and can be readily determined by the treating physician based on improvement in desired clinical symptoms.

In one exemplary embodiment, such methods involve administering BH4 in a dissolved form, wherein the formulation is dissolved in a liquid including but not limited to water, orange juice and apple juice. In one exemplary embodiment, dissolved BH4 is administered to the patient in a fasted condition, i.e., on an empty stomach. The invention further contemplates that the dissolved BH4, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal. Thus, BH4 may be ingested as a liquid product or pre-dissolved from a solid or semisolid dosage form prior to ingestion. In a further embodiment, BH4 may also be dissolved in the oral cavity from a solid or semisolid dosage form prior to swallowing the dissolved solution.

In another exemplary embodiment, such methods involve administering BH4 in a solid dosage form including but not limited to tablets, capsules, candies, lozenges, powders, and

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granules, or semisolid form, including but not limited to oral sprinkle into jelly, that is swallowed without dissolving in a liquid including but not limited to water, orange juice and apple juice, before swallowing. In one embodiment, swallowed BH4 is administered to the patient in a fasted condition, i.e. on an empty stomach. The invention further contemplates that the BH4 swallowed as a solid or semisolid dosage form, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal.

In another embodiment, such methods involve administering BH4, whether swallowed as a solid or semisolid dosage form, or dissolved in a liquid, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal. The invention further contemplates that BH4, whether swallowed or dissolved, is administered at a specified time including but not limited to morning, day, night, same time of the day, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal, one or more times a day. In an exemplary embodiment, BH4 is ingested once daily as a solid dosage form just after meals. In a preferred embodiment the solid dosage form is a formulated tablet or capsule. In more exemplary embodiments, BH4 is ingested within approximately 0 to 30 minutes, or 5 to 20 minutes, of eating a meal. Regardless of whether it is ingested as a solid dosage form, liquid dosage form or as a dissolved solution, the in vivo exposure (or bioavailability) of BH4 is higher when ingested just after meals compared to fasting controls.

The BH4 and the food may be ingested at approximately the same time, or the BH4 may be ingested before or after the food. The period of time between consuming the food and taking BH4, either swallowed or dissolved, may be at least 5 minutes. For example, BH4 may be administered 60 minutes, 30 minutes, 25 minutes, 20 minutes, 15 minutes, 10 minutes, or 5 minutes before or after a meal.

In another embodiment, for some patients, e.g. adults, or some disease states, e.g. cardiovascular diseases or other diseases associated with NOS dysfunction, the methods of the invention involve administering an intact tablet rather than dissolving the tablet in a liquid, in order to improve bioavailability.

In a second aspect, the invention contemplates a method of stabilizing BH4 in a patient's intestinal tract by decreasing intestinal pH, e.g. using proton exchange polymers. Corresponding products comprising BH4 and acidifying excipients, such as proton exchange polymers, are also contemplated.

A third aspect of the invention contemplates a method of increasing gut residence time for BH4, including but not limited to slowing of gut motility using an agent which slows gut motility, such as a fatty acid and/or a glycerol fatty acid ester. Such hydrophobic agents can increase the length of time that BH4 remains in the gut and can increase the amount of BH4 that gets absorbed. The length of time that BH4 remains in the gut, when formulated with such agent(s), can be at least one and a half times, at least two times, at least three times, at least four times, or at least five times longer than a BH4 formulation not having such an agent. Suitable fatty acids include oleic acid, stearic acid, arachidic acid, palmitic acid, arachidic acid, linoleic acid, linolenic acid, erucic acid, myristic acid, lauric acid, myristolic acid, and palmitolic acid. Also contemplated to increase gut residence time for BH4 is inducement of gastric retention using alginic acid, and

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bioadhesion using polycarbophil. Corresponding products comprising BH4 and agents that slow gut motility are contemplated.

A fourth aspect of the invention contemplates a method of modifying the release of BH4 using a sustained release formulation such as HPMC, carbomer, etc. Corresponding products that are sustained release formulations are contemplated.

In a fifth aspect, the invention contemplates administering BH4 in sterile liquid or sterile solid dosage form via routes other than oral administration including but not limited to topical, intravenous, subcutaneous, intramuscular, intrathecal, ophthalmic, and inhalational routes of administration. Corresponding compositions and kits suitable for such routes of administration, and methods of making the same, are contemplated. For example, a transdermal or buccal patch for transdermal or buccal administration, respectively, comprising BH4 is contemplated. Sublingual tablets comprising BH4 are also contemplated. Suitable kits are contemplated, including an inhaler device comprising BH4, or a kit comprising BH4 and a dropper or sprayer.

One embodiment includes a liquid formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, including an aqueous solution of BH4 or pharmaceutically acceptable salt thereof, an antioxidant, and a pH buffer.

Another embodiment includes a method of making a liquid formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof, including providing an aqueous solution containing BH4 or pharmaceutically acceptable salt thereof, adding an antioxidant and a pH buffer to the solution containing BH4 or pharmaceutically acceptable salt thereof, sparging the aqueous solution containing BH4 or pharmaceutically acceptable salt thereof, before or after addition of antioxidant and pH buffer, with an inert gas or carbon dioxide, and sealing the sparged solution containing BH4 or pharmaceutically acceptable salt thereof, antioxidant, and pH buffer in a container.

In a sixth aspect, the invention contemplates an improved method of measuring BH4 by utilizing tandem mass spectrometry and calculating the amount of reduced biopterin. Such methods can provide detection of BH4 to a sensitivity for BH4 in the range of 5-1000 ng/mL, with an accuracy and precision as exemplified by a coefficient of variation (CV) % below 15% (20% at the lower limit of quantitation, LLOQ). In an exemplary embodiment, a method of measuring BH4 using HPLC (RP) coupled with tandem mass spectrometry (LC/MS/MS) comprises the steps of: (1) subjecting samples of blood, plasma, tissue homogenates, or urine to oxidation; (2) subjecting the oxidized samples to iodometry; (3) passing said oxidized samples through an ion exchange column; (4) measuring total and oxidized biopterin in said samples using HPLC and tandem mass spectrometry; and calculating the amount of reduced biopterin as the difference between said total biopterins less said oxidized form. In one embodiment, samples are treated with acidic oxidation, wherein the method comprises the steps of (1) treating said samples with KCl, HCl or TCA; (2) subjecting said acid-oxidized samples to iodometry; (3) running said oxidized samples through an ion exchange column; (4) measuring total biopterin comprising 6R-BH4, R-q-DHBP (which is immediately reduced in vivo to 6R-BH4 such that the measured reduced biopterin is based mainly upon 6R-BH4), DHBP, and BP in said samples using HPLC and tandem mass spectrometry. In another embodiment, samples are treated by alkaline oxidation, wherein the method comprises: (1) treating said samples with KI, I or NaOH; (2) subjecting said alkaline oxidized samples to acidification with HCl or TCA; (3) subjecting said oxidized samples iodometry; (4) running said samples through an ion

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exchange column; (5) measuring oxidized biopterin comprising DHBP and BP using HPLC and tandem mass spectrometry; and (6) calculating the amount of reduced biopterin (6R-BH4+R-q-DHBP) as the difference between total biopterins less the oxidized form.

Another aspect of the invention is a mobile phase solution for reverse-phase HPLC separation of dihydrobiopterin, biopterin, and analogs thereof, including an aqueous solution including methanol, sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol. Similarly contemplated is a method of separating dihydrobiopterin and biopterin, or analogs thereof, from a mixture containing both base and dihydro forms, including performing reverse phase HPLC using a mobile phase comprising an aqueous solution including methanol, sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol, on a mixture containing dihydrobiopterin and biopterin, or an analog of dihydrobiopterin and an analog of biopterin.

Another aspect of the invention is a method of quantitating biopterins in a mixture of biopterin species, including providing a mixture comprising biopterin and at least one of dihydrobiopterin and tetrahydrobiopterin, or analogs of biopterin and at least one of dihydrobiopterin and tetrahydrobiopterin, separating the biopterin species in the mixture by reverse phase HPLC, and in the case of tetrahydrobiopterin and analogs thereof, performing electrochemical detection by oxidizing the tetrahydrobiopterin and analogs thereof present by a first electrode to quinonoid dihydrobiopterin forms, followed by reducing the quinonoid forms back to tetrahydrobiopterin and analogs thereof present at a second electrode, and measuring current generated by the reduction reaction to determine the concentration of species, and/or in the case of dihydrobiopterin, analogs thereof, biopterin, or analogs thereof, measuring such species by fluorescence detection following post-column oxidation of dihydrobiopterin species to biopterin.

For the compositions and methods described herein, preferred components, and compositional ranges thereof, can be selected from the various examples provided herein.

Other features and advantages of the invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a powder X-ray diffraction pattern characteristic of crystal polymorph form B of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin.

FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

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FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

FIG. 16 is a flow chart for the measurement of biopterin.

FIG. 17 is a summary of the validation of the biopterin assay.

FIG. 18 is a table showing pharmacokinetic parameters of total biopterins in plasma after a single oral administration of sapropterin (BH4) to rats.

FIG. 19 shows plasma biopterin concentration and reduced-form ratio after single-dose administration of sapropterin (BH4) to rats.

FIG. 20 shows plasma biopterin concentration and reduced-form ratio after a single-dose administration of sapropterin (BH4) in monkeys.

FIG. 21 is a table showing pharmacodynamic parameters of total biopterins in plasma after single-dose administration of sapropterin (BH4) to monkeys.

FIG. 22 shows the schedule of events for the evaluation of safety.

FIG. 23 shows the mean plasma concentrations of BH<sub>4</sub> after oral administration of 10 mg/kg of BH<sub>4</sub> as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers—linear axes.

FIG. 24 shows the mean plasma concentrations of BH<sub>4</sub> after oral administration of 10 mg/kg of BH<sub>4</sub> as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers—semi-logarithmic axes.

FIG. 25 shows a table summarizing the pharmacokinetic parameters for BH<sub>4</sub> after oral administration of 10 mg/kg of BH<sub>4</sub> as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers.

FIG. 26 shows a statistical comparison of pharmacokinetic parameters for BH<sub>4</sub> after oral administration of 10 mg/kg of BH<sub>4</sub> as dissolved and intact tablets under fasted conditions and intact tablets under fed conditions to healthy volunteers.

FIG. 27 shows a stability study of BH<sub>4</sub> formulated with 5% mannitol in an aqueous solution both before and after two weeks stored at -20° C.

FIG. 28 shows a dissolution profile of a BH<sub>4</sub> capsule formulation both before and after storage for 54 days at 40° C.



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FIG. 29 shows a dissolution profile of two BH4 formulations—a BH4 bioadhesive tablet and BH4 bioadhesive granules.

FIG. 30 shows a dissolution profile of various sustained release formulations of BH4.

FIG. 31 shows a dissolution profile of various sustained release formulations of BH4.

FIG. 32 shows a schematic diagram of a floating dosage formulations of BH4.

FIG. 33 shows a dissolution profile of various floating dosage formulations.

FIG. 34 shows a schematic diagram of gas generating dosage forms of BH4.

FIG. 35 shows a pharmacokinetic profile of various BH4 formulations.

FIG. 36 shows a stability study of intravenous BH4 formulations at pH 4 over 35 days.

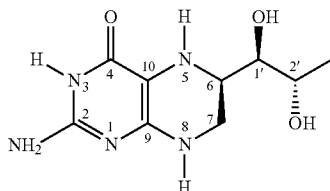
FIG. 37 shows a stability study of various intravenous BH4 formulations over 350 hours.

FIG. 38 shows a stability study of intravenous BH4 formulations at various BH4 concentrations.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides improved methods of orally administering a purified preparation of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin, including a pharmaceutically acceptable salt thereof. The invention is based on the finding that orally administered tetrahydrobiopterin (BH4) has low gastrointestinal absorption, which is a major contributing factor to the low bioavailability of BH4.

The chemical structure of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4) is shown below:



Tetrahydrobiopterin is a water soluble organic compound with low lipid solubility. Based on an in silico experimental analysis using BioLoom software (version 1.5 from Biobyte Corp in Claremont Calif), the octanol-water partition coefficient of BH4 was determined to be  $-1.17$ . Optimal penetration of biological membranes as approximated by the octanol/water partition coefficient occurs at around a log P of 2 or 100- $\times$  fold higher lipid solubility. Although a low ClogP allows this substrate to solubilize readily under physiological conditions, the ability of the substrate to penetrate bilipid layers within biological membranes is restricted, which may limit oral availability.

In vivo studies in rats and monkeys described herein showed that only 8-11% of BH4 is absorbed in the gut with the majority being excreted in the feces when compared to intravenous administration of BH4 at similar doses. Such variability in absorption of BH4 was also shown in a study described herein on the effect of food on the bioavailability of BH4 in healthy humans. Although the administration of BH4 in water and orange juice under fasted conditions resulted in comparable mean plasma concentrations and mean values for Cmax and AUC(0-t), the administration of BH4 concurrent

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with a high fat, high caloric meal resulted in a significant increase in the mean plasma concentrations and mean values for Cmax and AUC(0-t) when BH4 was administered in water.

Although there is ample literature describing increased bioavailability in fed conditions, this food effect is typically seen with lipophilic (i.e., lipid soluble) water-insoluble drugs and not usually with high water soluble active substance such as BH4. The usual explanation for increases in bioavailability under fed conditions for lipophilic compounds is that high fat meals help solubilize the drug since "like dissolves like" and this makes it available for absorption. Another possible explanation is that high fat meals stimulate the secretion of bile acids which are natural bio-surfactants that help solubilize and emulsify the fats we eat to aid their digestion. These bile acids are also thought to solubilize water-insoluble compounds thereby making them available for absorption. However, BH4 does not need solubilization to be absorbed since its solubility is greater than 1000 mg/mL and the compound is one of the most soluble drugs known. Therefore the enhancement of its bioavailability by high fat, high-energy meals is not consistent with such known mechanism.

However, administration as a solid or semi-solid dosage form and/or with a high-fat meal may maximize bioavailability by increasing the residence time of BH4 in the acidic milieu of the stomach and upper gastrointestinal tract (GIT) where BH4 is chemically stable. The stability of BH4 decreases with increasing pH and its half-life in pH 6.8 buffer solution, which is roughly the pH of the small intestine, is about 15 minutes. At pH 3.1, which is within realm of the typical pH of the stomach in normal volunteers, the stability of BH4 at a concentration of 1 mg/mL is over 3 hours. The chemical stability of BH4 may further increase when the pH of the stomach drops below pH 3.1. Therefore prolonged stomach residence time provides intact drug to the stomach wall for absorption, whereas rapid emptying into the intestine degrades BH4 and is thus not available to be absorbed.

Thus, to maximize oral bioavailability of BH4 at each administration, BH4 should be taken with food, e.g., a high fat food or a high fat and/or high calorie meal. Alternatively, to maximize consistency of oral bioavailability between administrations, BH4 should be taken on an empty stomach (e.g., 1 hour before or 2 hours after a meal).

As used herein, the term "bioavailability" refers to the fraction of an administered dose of a drug entering systemic circulation. If the drug were administered intravenously, then its bioavailability theoretically would be 100%. However, if the drug were administered via other routes (such as orally), then its bioavailability would be less than 100% as a result of, for example, incomplete absorption in the GI tract, degradation or metabolism prior to absorption, and/or hepatic first pass effect.

The term "high fat meal" refers generally to a meal of at least about 700 kcal and at least about 45% fat (relative percentage of kcal which are fat), or alternatively at least about 900 kcal and at least about 50% fat. The term "high fat food" refers generally to a food comprising at least 20 g of fat, or at least 25, 30, 35, 40, 45, or 50 g of fat, and/or at least about 45% or 50% fat. One FDA Guidance defines a "high-fat meal" as approximately 50% of total caloric content of the meal, whereas a "high-calorie meal" is approximately 800 to 1000 calories. The FDA recommends a high-fat and high-calorie meal as a test meal for food-effect bioavailability and fed bioequivalence studies. This test meal should derive approximately 150, 250, and 500-600 calories from protein, carbohydrate and fat, respectively. An example test meal consists of two eggs fried in butter, two strips of bacon, four

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ounces of hash brown potatoes and eight ounces of whole milk. Substitution is possible if a similar amount of calories from protein, carbohydrate, and fat has comparable meal volume and viscosity (Guidance for Industry, Food-Effect Bioavailability and Fed Bioequivalence Studies, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), December 2002).

In a first aspect, the invention provides methods of orally administering a purified preparation of 6R-(L-erythro)-5,6,7,8-tetrahydrobiopterin (BH4), including a pharmaceutically acceptable salt thereof.

In some embodiments, the methods involve informing the patient that administration of tetrahydrobiopterin with food has an effect on pharmacokinetics. In an exemplary embodiment, the methods comprise the step of informing the patient that absorption of tetrahydrobiopterin is increased when it is ingested with food compared to when ingested without food. In some embodiments, the patient is informed that ingestion shortly following a meal, for example, a high-fat, high-calorie meal, results in an increase in any one, two, three or all of the following parameters: mean plasma concentration, C<sub>max</sub>, AUC, AUC(0-t) and/or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 with a high-fat meal increases C<sub>max</sub> and AUC compared to administration of BH4 without food (in a fasting condition). In some embodiments, the relative increase can be at least 20% or 30% or more.

In alternative embodiments or in addition to the preceding embodiments, the method of administering tetrahydrobiopterin comprises informing the patient that absorption of tetrahydrobiopterin is increased when ingested as an intact tablet compared to when ingested after being dissolved in liquid. In some embodiments, the patient is informed that ingestion of intact tablets results in an increase in any of the following parameters: mean plasma concentration, C<sub>max</sub>, AUC, AUC(0-t) or AUC(inf). In exemplary embodiments, the patient is informed that administration of BH4 as an intact tablet increases C<sub>max</sub> and AUC compared to administration of BH4 after being dissolved in a liquid. In some embodiments, the relative increase can be at least 20% or more.

Any of the preceding methods may be carried out by providing or administering tetrahydrobiopterin in a container containing printed labeling informing the patient of the change in absorption parameters described above.

Optionally, the methods of the invention also comprise the step of providing to the patient in need thereof a therapeutically effective amount of tetrahydrobiopterin. The therapeutically effective amount will vary depending on the condition to be treated, and can be readily determined by the treating physician based on improvement in desired clinical symptoms.

In one exemplary embodiment, such methods involve administering BH4 in a dissolved form, wherein the formulation is dissolved in a liquid including but not limited to water, orange juice and apple juice. In one embodiment, dissolved BH4 is administered to the patient in a fasting condition, i.e., on an empty stomach. The invention further contemplates that the dissolved BH4, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, or 2.5 hours, or three hours after a meal. Thus, BH4 may be ingested as a liquid product or pre-dissolved from a solid or semisolid

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dosage form prior to ingestion. In a further embodiment, BH4 may also be dissolved in the oral cavity from a solid or semisolid dosage form prior to swallowing the dissolved solution.

These approaches maximize absorption rate and bioavailability by ensuring that BH4 is fully dissolved in solution or biologic fluids before it is delivered to its absorption sites, which are primarily the stomach and the intestine. Dissolution of active pharmaceutical ingredients or drug in solution is a prerequisite to absorption into the systemic (blood and lymphatic) circulation. When solid dosage forms such as tablets and capsules are administered orally, they go through a sequential series of steps such as disintegration into granules, de-aggregation into powders and dissolution prior to absorption into the systemic circulation. These series of steps are bypassed by administering liquid, semisolid and fast dissolving solid dosage forms. Thus the active substance is available earlier for absorption, and because there is no guarantee that a solid dosage form will release all the active substance contained within it before it transits through the absorptive sites, the formulations in which the active substance is present in dissolved form before it reaches the absorptive sites usually exhibits the greater bioavailability.

These dosage forms reduce variability in blood levels because the variability is dosage form disintegration and dissolution in vivo in the human is obviated. The rate of in vivo disintegration and dissolution of a solid dosage form of BH4 targeted for immediate-release in the stomach depends on the human-to-human variability in the pH of the gastric fluid—fed and unfed (fasting)—and the strength of the agitation intensity of the stomach as determined by the strength of gastric motility and gastric emptying rates into the small intestine. Since liquid, semisolid, lozenge/candy and fast dissolving solid dosage forms do not have to be subjected to disintegration and dissolution, their blood levels are less variable than when BH4 is given as immediate release solid dosage forms (tablets and capsules).

In another exemplary embodiment, such methods involve administering BH4 in a solid dosage form including but not limited to tablets, capsules, candies, lozenges, powders, and granules, or semisolid form, including but not limited to oral sprinkle into jelly, that is chewed or swallowed without dissolving in a liquid including but not limited to water, orange juice and apple juice, before swallowing. In one embodiment, swallowed BH4 is administered to the patient in a fasting condition, i.e., on an empty stomach. The invention further contemplates that the BH4 swallowed as a solid or semisolid dosage form, is administered at a specified time including but not limited to morning, day, night, same time of the day, on an empty stomach, one or more times a day. In exemplary embodiments, the composition is administered to the patient when the stomach is empty, for example, at least 30 minutes, 45 minutes, or at least one hour before, and/or at least 90 minutes, or two hours, 2.5 hours, or three hours after a meal.

In another embodiment, such methods involve administering BH4, whether swallowed as a solid or semisolid dosage form, or dissolved in a liquid, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal. The invention further contemplates that BH4, whether swallowed or dissolved, is administered at a specified time including but not limited to morning, day, night, same time of the day, with food, e.g. a high-fat food or a high-fat and/or high-calorie meal, one or more times a day. In an exemplary embodiment, BH4 is ingested once daily as a solid dosage form just after meals. In a preferred embodiment the solid dosage form is a formulated tablet or capsule. In more exemplary embodiments, BH4 is ingested within approximately 0 to 60 minutes, approxi-

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mately 0 to 30, or 5 to 20 minutes of eating a meal. Regardless of whether it is ingested as a solid dosage form, liquid dosage form or as a dissolved solution, the in vivo exposure (or bioavailability) of BH4 is higher when ingested just after meals compared to fasting controls.

The BH4 and the food may be ingested at approximately the same time, or the BH4 may be ingested before or after the food. The period of time between consuming food, e.g., a high-fat food or a high-fat and/or high-calorie meal and taking BH4 either swallowed or dissolved may be at least 5 minutes. BH4 may be administered 60 minutes, 30 minutes, 25 minutes, 20 minutes, 15 minutes, 10 minutes, or 5 minutes after ingestion of a meal.

In another embodiment, for some patients, e.g. adults, or some disease states, e.g. cardiovascular diseases or other diseases associated with NOS dysfunction, the methods of the invention involve administering an intact tablet rather than dissolving the tablet in a liquid, in order to improve bioavailability.

Administration of BH4 according to the methods of the invention results in mean plasma concentrations and/or rate of gastrointestinal absorption and/or mean values for Cmax and/or AUC(0-t) and/or AUC (inf) that exceeds the values when BH4 is administered under fasted conditions.

Administration of an intact tablet under fasted conditions resulted in an average 20% increase in Cmax and AUC relative to dissolved tablets. Administration of a dissolved tablet in either water or orange juice or an intact tablet after a high fat/high calorie meal resulted in increases in Cmax and AUC that ranged from approximately 30% (intact tablet) to 80% (water). Administration of BH4 as an intact tablet following a high fat and high calorie meal resulted in an approximate 30% increase in the extent of absorption compared to administration without food. Administration of BH4 as an intact tablet resulted in an approximate 20% increase in the extent of absorption compared to administration of dissolved tablets.

"Mean plasma concentration" means the average of readings of concentration in a series of plasma samples.

"Cmax" means the maximum observed plasma concentration.

"AUC" means the area under the plasma concentration-time curve.

"AUC<sub>0-t</sub>" means the area under the plasma concentration-time curve from time 0 to the time of the last measurable concentration.

"AUC<sub>(inf)</sub>" means the calculated area under the plasma concentration-time curve from time 0 to infinity.

The "rate of gastrointestinal absorption" of BH4 is estimated from the area under the plasma total biopterin concentration increase (ΔCp)-time curve (ΔAUC) after the administration of BH4 using the following formula:

$$\text{Absorption rate(\%)} = \frac{(\Delta\text{AUC after p.o. dose}/\Delta\text{AUC after i.v. dose}) \times (\text{i.v. dose}/\text{p.o. dose} \times 100)}{1}$$

Preferably at least 99.5% pure 6R-BH4 is used. Any salt, including the dihydrochloride salt, and any crystalline form of BH4 may be utilized according to the methods and compositions of the invention. A variety of salts and crystalline forms are described in U.S. Patent Publication No. 2006/0040946, incorporated herein by reference in its entirety, and/or the stable solid formulation described in Int'l Publication No. WO 06/55511, also incorporated herein by reference in its entirety. The various crystalline forms may conveniently be formed into a tablet, powder or other solid for oral administration.

In a second aspect, the invention contemplates a method of stabilizing BH4 by decreasing intestinal pH using proton

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exchange polymers. BH4 is administered orally daily as a solid or liquid dosage form comprising inactive ingredients that enhance the stability of BH4 beyond the stomach by lowering the pH of the intestine and thus preserving BH4 from being oxidized rapidly. Since BH4 is more stable in acidic media than in basic media, acidifying excipients/inactive ingredients are included in solid dosage (tablets, capsules, etc) formulations of BH4 to lower the pH of the intestinal fluids and thereby enhance the chemical stability. The larger area or window of the gastrointestinal tract (GIT) available for absorption optimizes the consistency of absorption by expanding the current limited window of absorption believed to be limited to the stomach and the duodenum to the intestine. Such dosage forms include but are not limited to effervescent tablets, powders and granules (to be resuspended in liquid before administration) and acidifier materials. Unlike small molecule acids, bulky polymeric acids remain in the GIT longer and are not absorbed by the GIT, but donate their protons to the GIT fluids to lower the environmental pH. Examples of excipients/inactive ingredients that comprise the formulation are carboxylic acid small molecules such as maleic, fumaric and citric acids or inorganic small molecules such as phosphoric acid, acetic acid and their salt forms. Other examples are pharmaceutically acceptable acids such as polymeric carboxylic acid classes including polymethacrylic acids, carbomers, polycarbophil, Eudragits, acid forms of crosscarmellose and starch glycolic acid, etc. The formulations also contain additional excipients to enhance stability such as anti-oxidants (e.g., thiols such as cysteine, N-acetyl cysteine, etc; ascorbic acid; methionine; etc.) and other excipients known in the trade to enable manufacturability and enhance the quality and performance attributes of the formulation.

A third aspect of the invention contemplates a method of increasing gut residence time for BH4, including but not limited to slowing of gut motility using an agent which is capable of slowing gut motility of BH4, such as a fatty acid and/or a glycerol fatty acid ester. Fatty acids can include oleic acid, stearic acid, arachidic acid, palmitic acid, archidoic acid, linoleic acid, linolenic acid, erucic acid, myristic acid, lauric acid, myristolic acid, and palmitolic acid. Also contemplated for increasing gut residence time of BH4 are the inducement of gastric retention using alginic acid and bioadhesion using polycarbophil. In one embodiment, dosage forms of BH4 are administered as oral buoyant formulations that float and release BH4 in a defined fashion in the gastric fluid and are retained longer in the stomach because they are more resistant to gastric emptying from the stomach than formulations that are non-buoyant or dissolve rapidly in the stomach. This design approach is based on gastro-retention of the dosage form via the use of a gas-generating excipient within the dosage form, low-density excipients that render the dosage form buoyant in GIT fluids or a combination of a gas and low-density materials in a dosage form to enable the floating of the dosage form in the fluid contents of the GIT. Prolonged retention and release of the dosage form in the stomach milieu wherein BH4 is more stable in its acidic fluids will enhance both residence time of the dosage form in the stomach and the stability of BH4 and thus make BH4 available for a longer period absorption in the stomach and duodenum than standard tablet and capsule dosage forms. Formulations of BH4 will comprise of one or more antioxidants, excipients known in the field to enable manufacturing and disintegration/dissolution of the solid dosage form and additional excipients that generate a gas or mixture of gases (e.g., carbon dioxide) upon contact of the formulation with aqueous media and or GIT fluids. Water-soluble antioxidants are pre-



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ferred, for example, ascorbic acid, methionine, and thiols (cysteine, N-acetyl cysteine and glutathione) or anti-oxidants that are converted to a soluble antioxidant in the GIT, e.g., ascorbyl palmitate which is converted to ascorbic acid in the GIT. Excipients added to the formulation include carbonates and bicarbonates that react directly with BH4 to form carbon dioxide and small and polymeric acids described previously to react with the carbonates and bicarbonates to produce additional carbon dioxide as needed.

In another embodiment, dosage forms of BH4 are administered that adhere for a prolonged time to the mucous surfaces of the GIT (i.e., bioadhesive formulation), preferably in, but by no means limited to the stomach where due to the acidity of gastric fluids, BH4 is more stable than in the intestine. BH4 is released in a controlled manner from the bioadhesive dosage form. The solid dosage form is designed to contain BH4, one or more antioxidants, excipients known in the field to enable the manufacturing of quality dosage forms and control the disintegration/dissolution of the dosage form and a bioadhesive additive such as polycarbophil in its free acid form or as a salt form. Other polymeric acids such as polymethacrylic acids, carbomers and cellulose derivatives, e.g., HPMC, HPC, etc. may be combined with or substituted for polycarbophil. The antioxidants are preferably soluble, for example, ascorbic acid, methionine, cysteine, N-acetyl cysteine and glutathione or can be converted to a soluble antioxidant such as ascorbic acid in the GIT, e.g., ascorbyl palmitate. In one embodiment, the components of the formulation are blended together and manufactured as a solid dosage form, e.g., tablets or capsules. The solid dosage form may be enteric coated to deliver BH4 past the stomach into the intestine or not enteric coated designed to release BH4 in the stomach. In another embodiment, the components of the solid dosage form may be subdivided into different portions and the various portions are blended separately before they are processed to form multilayered dosage forms. The multilayered dosage form may contain the bioadhesive and a few excipients in the outermost layer of a tablet, wrapped around other layers that contain BH4 (i.e., active region inside a bioadhesive envelope) or as a wrap-around cylindrical plug filled into a capsule wherein one or more other layers are assembled beneath or within the bioadhesive envelope. Alternatively, the bioadhesive and other layers in the tablet or capsule plugs may be layered in a parallel bi- or multilayer configuration. These designs allow the bioadhesive to interact with the GI membrane or GI membrane mucus to anchor the dosage form to the membrane slowing down its transit through the GI tract and thus increasing residence time. Such dosage forms may also be enteric coated. Yet another embodiment of the method used with BH4 is to employ polymeric inactive ingredients (excipients) with functional groups that bind to GIT mucus to delay the transit of the dosage form through the GIT. Dosage forms of BH4 are formulated with thiolated polymer excipients (polymer-SH) such as polycarbophil-cysteine, polypolymethacrylic acid-cysteine, carboxymethyl cellulose-cysteine, chitosan derivatives-cysteine, etc. These thiolated polymers confer both bioadhesive and anti-oxidant properties on BH4 considerably enhancing absorption. Other excipients included in these formulations are antioxidants and performance and manufacture-aiding excipients.

In yet another embodiment, oral dosage forms containing inactive excipients or active ingredients are used to slow gastric motility. Slowing down the transit of BH4 dosage form through the GIT tract will increase the residence time of the molecule and thus enable a larger fraction of the administered dose to be absorbed. Generally regarded as safe

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(GRAS) excipients employed in oral formulations to delay gastric emptying and/or delay intestinal motility preferably comprise dietary fats such as fatty acids, glycerides of fatty acids, and derivatives of fatty acids and glycerides such as Cremophor™ (polyoxyl castor oil derivatives), etc. Active excipients include agents that slow gut motility such as general or selective ( $M_3$ ) antimuscarinic or anticholinergic agents.

A fourth aspect of the invention contemplates a method of modifying the release of BH4 using a sustained release formulation such as HPMC, carbomer, etc. This concept comprises delivering BH4 dosage forms to the GI tract by modifying or altering the release of BH4 from immediate-release to slow, prolonged, controlled and or timed release. Slow, prolonged and controlled release is achieved using excipients known in the art and BH4 is protected within the delivery system from chemical degradation by the presence of stability enhancers such as anti-oxidants. Such methods can maximize bioavailability since BH4 is stabilized within the formulation and in the environment surrounding the formulation to enable the active molecule to be absorbed intact into the systemic circulation as the formulation transits the entire length of the GIT. This approach provides a larger window of the GIT for absorption and does so by preventing the degradation of BH4 in the higher pH milieu so that BH4 is available to be absorbed. Antioxidants will be included in the formulation to protect the drug from degrading in intestinal fluids due to near neutral pH of the intestinal fluids. Slow, prolonged and controlled delivery will also deliver BH4 to low oxygen tension regions of the GIT. Timed release is achieved using excipients known in the art such as pH sensitive polymers that dissolve only when the pH reaches a value wherein the polymer is soluble.

In another embodiment, the invention contemplates enteric coating of the BH4 dosage form to ascertain whether including acidic excipients in a formulation of BH4 does indeed increase absorption of BH4 by lowering the pH of the intestine and thus stabilizing BH4 in the intestine to be available for absorption. Thus, enteric coating will be used to keep the excipients and drug together at the site where the excipient is expected to protect BH4. If the BH4 dosage form were allowed to disintegrate in the stomach, the acidic excipients may not empty together into the stomach and may not provide protection.

Enteric coating protects compounds susceptible to acid-catalyzed degradation in the stomach from getting degraded by the acid in the stomach. Enteric coating materials prevent the tablet or capsule from releasing the active compound in the stomach because the enteric coating materials are insoluble in acid. Once the enteric-coated dosage form reaches the intestine where the pH value varies from pH 5-8, the materials become soluble and release the active substance in the intestine. In contrast, sustained release formulations are designed to release medicaments over as long a length/area of the GIT as possible. Coating a sustained release formulation to release just past the stomach may be necessary only if the medicaments contained in it are acid-labile.

In a fifth aspect, the invention contemplates administering BH4 in sterile liquid or sterile solid dosage form via routes other than oral administration including but not limited to topical, intravenous, subcutaneous, intramuscular, intrathecal, ophthalmic, and inhalational routes of administration. BH4 is formulated as a sterile liquid or solid dosage form at the appropriate concentration desired.

The advantages of a sterile liquid dosage form of BH4 for intravenous administration may include: (1) more predictable kinetics, with the potential for higher serum levels; (2) no

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requirement of a functional gastrointestinal tract; (3) no requirement for patient participation; and (4) absence of a noncompliance concern. Intravenous formulations of BH4 may be particularly beneficial in managing conditions requiring expedited delivery of fluids and medications throughout the body or to body compartments normally difficult to access via oral or other forms of administration, including but not limited to rabies, meningitis, organ transplantation/preservation, sub-arachnoidal hemorrhages, brain trauma, stroke, coronary artery bypass surgery, cerebrovascular vasospasm, blood transfusion/preservation, pulmonary hypertension, sickle cell disease, pre-eclampsia, and post-chemotherapy vascular disease.

BH4 is highly susceptible to oxidation in aqueous solution and in physiologic aqueous pH solutions (Davis, et al., Eur. J. Biochem. 173, 345-351 (1988); Kirsch, et al., J. Biol. Chem. 278, 24481-24490 (2003)). Most determinations of BH4 stability have been carried out in neutral to mildly alkaline pH 7.4 solutions to mimic the likely stability behavior of BH4 under physiologic plasma pH condition. Although European Patent Application No. 1 757 293 A discloses liquid or syrup formulations, such formulations consist of solid state powder blends or granulations that require reconstitution with water prior to oral ingestion. The present aspect of invention contemplates liquid formulations not limited to powders or granulations for constitution. The invention also contemplates compounded liquid formulations able to remain stable at ambient temperature for a sufficient period of time to allow processing in sterile product fill/finish facilities to be filled into ampoules, bottles or vials as a liquid product or filled into vials to be freeze-dried into lyophilized products.

The liquid and lyophilized formulations for reconstitution can also be delivered via the nasal, ophthalmic and ear canal for therapeutic effects. The formulation of a lyophilized product requires prior dissolution of BH4 in a liquid, preferably aqueous, and the processing of the liquid product in a sterile facility (i.e. compounding, sterile filtration and filling of the sterile-filtered liquid into vials prior to the loading of the filled vials into a lyophilizer for lyophilization). Maintaining the stability of solubilized BH4 during sterile processing and preventing its degradation are key prerequisites to manufacturing lyophilized product that satisfies impurities specification for the fill-finished product. Therefore the composition of the lyophilized product contains appropriate stabilizers that minimize or obviate BH4 degradation during the fill finish process. The formulations described herein would stabilize BH4 solutions during sterile fill/finish manufacturing, a process that takes a minimum of six hours, and also provide commercially stable product.

The formulations include BH4, preferably in concentration in a range of 0.1 mg/mL to 10 mg/mL. Due to the high solubility of BH4, formulations with concentrations up to about 100 mg/mL, for example, can also be prepared. The general relative compositional makeup and methods described herein are applicable for making highly concentrated solutions.

Liquid formulations of BH4 preferably are formulated in pH 1 to 8 buffer solutions, preferably in pH 2 to 7 buffer solutions. The pH buffers chosen are buffer compounds capable of providing substantial buffering capacity at a particular pH desired, as judged by how close the buffer ionization constant or constants are to the desired pH of the liquid formulation. Thus any buffer compounds may be employed as long as one or more of the compound's ionization constants are close to the desired pH of the formulation. Examples of buffers that may be employed in the pH 1-8 range comprise various acids/bases and their respective conjugate acids/bases

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or salt forms, including but not limited to: hydrochloric acid (pH 1-2), maleic acid (pH 1-3), phosphoric acid (pH 1-3), citric acid (pH 3-6), acetic acid (pH 4.7±1.0), sodium phosphate dibasic (pH 6-8), tromethamine (TRIS, pH 8.3±1.0), and the like.

## Intravenous Formulations

Intravenous formulations are stabilized using an anti-oxidant or a combination of 2 or more antioxidants. Combinations of anti-oxidants can be synergistic in obviating instability of the formulation. Sparging with inert gases and or carbon dioxide to remove dissolved oxygen from solution is optional, but is preferred when low concentrations of antioxidants are used, and further preferably when both low concentrations of BH4 and antioxidants are used. Stabilization of BH4 in aqueous solution is influenced by the interactions of the concentration of BH4 with the antioxidant and pH. Thus, for example, high concentrations of BH4 require less antioxidant concentrations than low concentrations of BH4. Furthermore, BH4 is more stable at low pH than at high pH. Therefore desired high pH formulations preferably have higher antioxidant concentrations, more preferably a combination of 2, 3, or more antioxidants, and still further preferably sparging with non-oxidizing gas (e.g., inert gas or carbon dioxide) followed by hermetically or near-hermetically sealing the primary container in an atmosphere of a non-oxidizing gas (e.g., inert gas or carbon dioxide) to further enhance the stability of the drug product.

Example ranges for BH4 liquid formulations are given in Tables 1 and 2. Formulated or compounded solutions are optionally sparged with an inert gas (e.g., argon or nitrogen) or carbon dioxide in the compounding tank and primary containers preferably are sealed in a blanket of inert gas or carbon dioxide to remove oxygen from the container headspace. The formulation can be scaled up to any volume by multiplying the component amounts by an appropriate scale up factor.

TABLE 1

General examples of composition ranges  
in a low pH (e.g., pH 4.0) formulation

Components	Amount (mg)	% Weight/ Volume	Function
BH4	0.10-100	0.01-10.00	Active substance
L-Cysteine	0.00-50.00	0.00-5.00	Antioxidant
Ascorbic Acid	0.00-500.00	0.00-50.00	Antioxidant
Sodium Metabisulfite	0.00-300.00	0.00-30.00	Antioxidant
Citric Acid	0.26-19.87	0.03-1.99	Buffering agent
Sodium Citrate, Dihydrate	2.57-192.75	0.26-19.27	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 2

General examples of composition ranges of a neutral pH  
(e.g., pH 7.0) formulation

Components	Amount (mg)	% Weight/ Volume	Function
BH4	0.10-100	0.01-10.00	Active substance
L-Cysteine	0.00-50.00	0.00-5.00	Antioxidant
Ascorbic Acid	0.00-500.00	0.00-50.00	Antioxidant
Sodium Metabisulfite	0.00-300.00	0.00-30.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	0.50-11.02	0.05-1.02	Buffering agent
Sodium Dibasic Phosphate	0.44-17.80	0.04-1.78	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

The antioxidants employed for liquid formulations preferably are selected from one or more of thiol-based (e.g., L-cysteine), ascorbic acid and sulfite-based (e.g. sodium metabisulfite) compounds. Solutions preferably are sparged with inert gases or carbon dioxide to expel oxygen from the BH4 solutions and then hermetically sealed in ampoules or hermetically capped vials and bottles using metallic beverage beer-type caps in a blanket of inert gases (e.g., argon, nitrogen) or non-inert gas such as carbon dioxide to keep the sparged gases in the container head spaces from escaping. Oral liquid formulations preferably additionally contain sweeteners and flavorants improve the palatability of the formulations.

In one embodiment, as a liquid dosage form, BH4 is stabilized by anti-oxidants and/or by sparging with non-oxidizing, preferably sterilized, gases, such as inert gasses (e.g., nitrogen, argon, helium, etc.) and/or a non-inert gas such as carbon dioxide to remove molecular oxygen from the formulation. The product is preferably filled under a blanket of inert gasses to minimize or prevent molecular oxygen from redissolving in the formulation. The liquid is filled into a container (e.g., vials, ampoules, etc.) and hermetically sealed to prevent oxygen from entering the container. In another embodiment, as a sterile solid dosage form for parenteral administration, a solution of BH4 is lyophilized and reconstituted in the clinic prior to administration. In yet another embodiment, sterile powder drug substance of BH4 is directly packaged into sterile containers (e.g., vials, bags, bottles or ampoules) in a sterile dry powder fill facility. Thus, another aspect of the invention is a dry powder formulation of tetrahydrobiopterin (BH4) or a pharmaceutically acceptable salt thereof for constitution into an aqueous solution, including a dry powder mixture of BH4 or pharmaceutically acceptable salt thereof, an antioxidant, and a pH buffer.

#### Oral Liquid Formulation Compositions

Oral liquid formulations comprise in addition to the components employed in the general liquid and intravenous formulations, sweeteners and flavoring agents. Sweeteners and flavors are added in quantities sufficient to yield acceptable sweetness and flavor. Oral liquid formulations contain one or more stabilizers. Optionally, they contain antimicrobial preservatives. They are preferentially buffered at low pH e.g., pH 1-4 and the buffering agents are selected to match the flavoring agent thus enhancing the organoleptic properties of the oral liquid formulation. Examples of preferred buffers (acid and conjugate bases) are: citric acid, tartaric acid, malic acid in combination with their conjugate bases or salt forms.

Examples of sweeteners include sugars (e.g., sucrose, glucose, sorbitol, mannitol, fructose, etc.), intense non-sugar sweeteners (e.g., aspartame, acesulfame K, cyclamate, saccharin, sucralose, glycyrrhizin, alitame, neotame, neohesperidine DC, thaumatin, monellin, and the like).

In a further embodiment, for nasal, ophthalmic and otic administrations, BH4 is formulated as discussed for parenteral dosage forms and is optionally a sterile product. These dosage forms can be provided in a kit package presentation with several days of supplies. Each unit within the kit can be comprised of one vial or ampoule and one sprayer (for nasal dosage form) or one dropper (in the case of ophthalmic and otic dosage forms). Once the vial or ampoule is opened, the sprayer or dropper is screwed onto the vial or ampoule and the previous cap is discarded. The dosage form product is used within a prescribed expiration period and discarded and a new vial or ampoule is opened for use. Another embodiment is to fill the solutions in hermetic plastic single-use disposable sterile containers produced by a form-fill-and-seal manufacturing process. These packages are opened and the solutions

delivered using the desired route of administration by squeezing out the liquid contained within them. These dosage forms are administered once daily and are given via the nostrils (nasal product), or via the eyes (ophthalmic) or droplets are instilled into the auditory canal (otic product). With respect to medication packaged in form, a fill and seal package, the medication is squeezed out onto the route of administration.

In a further embodiment, BH4 is administered via buccal and transdermal routes using formulated strips, patches or films or as topical products that placed on the site of delivery. Sublingual tablets are placed beneath the tongue. These dosage forms are administered once daily and are either attached to the delivery site membrane (buccal and transdermal route) or placed as a solid or semi-dosage form in the sublingual site. To prevent irritation of the delivery site, a basic compound such as sodium carbonate or bicarbonate is coated and mixed with BH4 to prevent interaction with BH4 that would render it unstable. Alternatively the basic compound is added just before use to raise the pH of BH4, which is quite low. Adding the basic excipient at the time of manufacturing without coating the alkaline particles to prevent interaction with BH4, will lead to instability of BH4. Another embodiment is to coat a core sublingual tablet of BH4 with a coating solution containing a basic or alkaline substance. In the sublingual compartment, the basic compound dissolves first, and interacts with BH4 to raise the pH of the medium.

#### Primary Container Packaging for BH4 Liquid Formulations

The primary packaging containers for BH4 liquid formulations are preferably impermeable to oxygen, carbon dioxide, nitrogen and inert gases. Following filling of sparged liquid formulations of BH4 into the primary container, preferably under a blanket of nitrogen, the containers are preferably hermetically sealed to keep the sparging gas in the liquid and container headspace and prevent the loss of the sparging gas and ingress of oxygen into the container.

The preferred primary containers are hermetically sealed ampoules as well as bottles and vials sealed hermetically with metallic cap such as those employed in sealing soda and beer beverage bottles. During use, the ampoules are cut opened and used within a few hours, e.g., about 12 hours. Ampoules can be used for intravenous and sterile products for injections. Sterile injectable liquids and lyophilized products can also be packaged in rubber closure-sealed vials which are secured with crimped aluminum cap. The antioxidants in the formulations protect the liquid and lyophilized products from the imperceptibly slow loss of sparged gas or oxygen ingress into the vial for the shelf life of the product.

BH4 liquid formulations filled into bottles or vials for oral, ophthalmic or otic use preferably are hermetically secured with a beverage metallic cap or a rubber stopper secured with crimped aluminum seal. The flutes of the bottles or vials can be grooved to accept a screw cap. When the hermetic seal is removed, it is replaced with a screw cap with or without a dropper. The presence of antioxidants in the formulation can enable the screw-capped formulation to be stable for use for at least two weeks, for example, after the hermetic seal is broken.

#### I. Synthesis of Tetrahydrobiopterin

A variety of methods are known in the art for synthesis of tetrahydrobiopterins, precursors, derivatives and analogs. U.S. Pat. Nos. 5,698,408; 2,601,215; 3,505,329; 4,540,783; 4,550,109; 4,587,340; 4,595,752; 4,649,197; 4,665,182; 4,701,455; 4,713,454; 4,937,342; 5,037,981; 5,198,547; 5,350,851; 5,401,844; 5,698,408, Canadian application CA 2420374, European application nos. EP 079 574, EP 191 335 and Suntory Japanese patent publications JP 4-082888, JP 59-021685 and JP 9-157270, as well as Sugimoto and Mats-



uura, Bull. Chem. Soc. Japan, 48(12):3767-3768 (1975), Sugimoto and Matsuura, Bull. Chem. Soc. Japan, 52(1):181-183 (1979), Matsuura et al., Chem. Lett. (Japan), 735-738 (1984), Matsuura et al., Heterocycles, Vol. 23, No. 12, 3115-3120, 1985 and Whiteley et al., Anal Biochem. 137(2):394-6 (1984) (each incorporated herein by reference) each describe methods of making dihydrobiopterins, BH4 and derivatives thereof that may be used as compositions for the present invention.

Int'l Publication No. WO2005049614, U.S. Pat. No. 4,540, 783, Japanese Patent No. 59-021685, Schircks et al., Helv. Chim. Acta, 60: 211 (1977), Sugimoto et al., Bull. Chem. Soc. Jp, 52(1):181 (1979), Sugimoto et al., Bull. Chem. Soc. Jp, 48(12):3767 (1975), Visontini et al., Helv. Chim. Acta, 52:1225 (1969), and Matsuura et al., Chem. Lett., p 735 (1984), incorporated herein by reference in their entirety, describe methods of synthesizing BH4.

## II. Crystalline Forms Of 6R-tetrahydrobiopterin Hydrochloride Salt

(6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists in different crystalline forms, including polymorphic forms and solvates, some of which are more stable than others.

### Crystal Polymorph Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

#### Polymorph Form B

The crystal polymorph that has been found to be the most stable is referred to herein as "form B," or alternatively as "polymorph B." Results obtained during investigation and development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride development revealed that there are several known crystalline solids have been prepared, but none have recognized the polymorphism and its effect on the stability of the BH4 crystals.

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties render polymorph form B especially feasible for pharmaceutical application, which are prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

Form B exhibits an X-ray powder diffraction pattern, expressed in d-values (Å) at: 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). FIG. 1 is a graph of the characteristic X-ray diffraction pattern exhibited by form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

As used herein, the following the abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity. A characteristic X-ray powder diffraction pattern is exhibited in FIG. 1.

It has been found that other polymorphs of BH4 have a satisfactory chemical and physical stability for a safe handling during manufacture and formulation as well as providing a high storage stability in its pure form or in formulations. In addition, it has been found that form B, and other polymorphs of BH4 can be prepared in very large quantities (e.g., 100 kilo scale) and stored over an extended period of time.

All crystal forms (polymorphs, hydrates and solvates), inclusive of crystal form B, can be used for the preparation of

the most stable polymorph B. Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms than polymorph form B, such as polymorph A, in suitable polar and non aqueous solvents. Thus, the pharmaceutical preparations described herein refer to a preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Other forms of BH4 can be converted for form B by dispersing the other form of BH4 in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce polymorph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B. Ambient temperatures, as used herein, mean temperatures in a range from 0° C. to 60° C., preferably 15° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents for the conversion of other forms to form B include but are not limited to, methanol, ethanol, isopropanol, other C3- and C4-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methyl-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C3-C6-acetates, methyl ethyl ketone and other methyl-C3-C5 alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours or less than 20 hours.

Polymorph B may also be obtained by crystallization from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. It has been found that polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals. Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water:acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallization from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallization and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In one embodiment of the compositions and methods described herein, a composition including polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is prepared by dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals. The composition is further modified into a pharmaceutical composition as described below.

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The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, by reference to the solution. Preferred non-solvents (i.e., solvents useful in preparing suspensions of BH4) are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described above.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties render polymorph form B especially feasible for pharmaceutical application.

#### Polymorph Form A

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form A," or "polymorph A." Polymorph A is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph A is a hygroscopic anhydrate, which is a meta-stable form with respect to form B; however, it is stable over several months at ambient conditions if kept in a tightly sealed container. Form A is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form A can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Polymorph A which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) of: 15.5 (vs.), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m), 3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w). FIG. 2 is a graph of the characteristic X-ray diffraction pattern exhibited by form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph A exhibits a characteristic Raman spectra bands, expressed in wave numbers (cm<sup>-1</sup>) at: 2934 (w), 2880 (w), 1692 (s), 1683 (m), 1577 (w), 1462 (m), 1360 (w), 1237 (w), 1108 (w), 1005 (vw), 881 (vw), 813 (vw), 717 (m), 687 (m), 673 (m), 659 (m), 550 (w), 530 (w), 492 (m), 371 (m), 258 (w), 207 (w), 101 (s), 87 (s) cm<sup>-1</sup>.

Polymorph form A may be obtained by freeze-drying or water removal of solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water. Polymorph form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at ambient temperatures in water, (1) cooling the solution to low temperatures for solidifying the solution, and removing water under reduced pressure, or (2) removing water from said aqueous solution.

The crystalline form A can be isolated by filtration and then dried to evaporate absorbed water from the product. Drying conditions and methods are known and drying of the isolated product or water removal pursuant to variant (2) described herein may be carried out in applying elevated temperatures, for example up to 80° C., preferably in the range from 30° C. to 80° C., under vacuum or elevated temperatures and vacuum. Prior to isolation of a precipitate obtained in variant (2), the suspension may be stirred for a certain time for phase

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equilibration. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 5 to 40 percent by weight, by reference to the solution.

A fast cooling is preferred to obtain solid solutions as starting material. A reduced pressure is applied until the solvent is completely removed. Freeze drying is a technology well known in the art. The time to complete solvent removal is dependent on the applied vacuum, which may be from 0.01 to 1 mbar, the solvent used and the freezing temperature.

Polymorph form A is stable at room temperature or below room temperature under substantially water free conditions, which is demonstrated with phase equilibration tests of suspensions in tetrahydrofuran or tertiary-butyl methyl ether stirred for five days and 18 hours respectively under nitrogen at room temperature. Filtration and air-drying at room temperature yields unchanged polymorph form A.

#### Polymorph Form F

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form F," or "polymorph F." Polymorph F is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph F is a meta-stable form and a hygroscopic anhydrate, which is more stable than form A at ambient lower temperatures and less stable than form B at higher temperatures and form F is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form F can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 μm to about 500 μm.

Polymorph F exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 17.1 (vs.), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w), 3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 296 (m). FIG. 3 is a graph of the characteristic X-ray diffraction pattern exhibited by form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph F may be obtained by phase equilibration of suspensions of polymorph form A in suitable polar and non-aqueous solvents, which scarcely dissolve said lower energy forms, especially alcohols such as methanol, ethanol, propanol and isopropanol. Polymorph form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can also be prepared by dispersing particles of solid form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-aqueous solvent that scarcely dissolves said (6R)-L-erythro-tetrahydrobiopterin dihydrochloride below room temperature, stirring the suspension at said temperatures for a time sufficient to produce polymorph form F, thereafter isolating crystalline form F and removing the solvent from the isolated form F. Removing of solvent and drying may be carried out under air, dry air or a dry protection gas such as nitrogen or noble gases and at or below room temperature, for example down to 0° C. The temperature during phase equilibration is preferably from 5 to 15° C. and most preferably about 10° C.

#### Polymorph Form J

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form J," or "polymorph J." The polymorph J is slightly

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hygroscopic and adsorbs water when handled at air humidity. The polymorph J is a meta-stable form and a hygroscopic anhydrate, and it can be transformed back into form E described below, from which it is obtained upon exposure to high relative humidity conditions such as above 75% relative humidity. Form J is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form J can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form J exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs). FIG. 4 is a graph of the characteristic X-ray diffraction pattern exhibited by form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph J may be obtained by dehydration of form E at moderate temperatures under vacuum. In particular, polymorph form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by taking form E and removing the water from form E by treating form E in a vacuum drier to obtain form J at moderate temperatures, which may mean a temperature in the range of 25 to 70° C., and most preferably 30 to 50° C.

#### Polymorph Form K

It has been found that another crystal polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form K," or "polymorph K." Polymorph K is slightly hygroscopic and adsorbs water to a content of about 2.0 percent by weight, which is continuously released between 50° C. and 100° C., when heated at a rate of 10° C./minute. The polymorph K is a meta-stable form and a hygroscopic anhydrate, which is less stable than form B at higher temperatures and form K is especially suitable as intermediate and starting material to produce stable polymorph forms, in particular form B. Polymorph form K can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form K exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m). FIG. 5 is a graph of the characteristic X-ray diffraction pattern exhibited by form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Polymorph K may be obtained by crystallization from mixtures of polar solvents containing small amounts of water and in the presence of small amounts of ascorbic acid. Solvents for the solvent mixture may be selected from acetic acid and an alcohol such as methanol, ethanol, n- or isopropanol. In particular, polymorph form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and an alcohol or tetrahydrofuran containing small amounts of water and a small amount of ascorbic acid at elevated temperatures, lowering temperature below room temperature to crystallize said dihydrochloride, isolating the precipitate and drying the isolated precipitate at elevated temperature optionally under vacuum. Suitable alcohols are for example methanol, ethanol, propanol and isopropanol, whereby ethanol is preferred. The ratio of acetic acid to alcohol or tetrahydrofuran may be from 2:1 to 1:2 and preferably about 1:1. Dissolution of (6R)-L-erythro-tetrahydro-

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biopterin dihydrochloride can be carried out in presence of a higher water content and more of the anti-solvent mixture can be added to obtain complete precipitation. The amount of water in the final composition may be from 0.5 to 5 percent by weight and the amount of ascorbic acid may be from 0.01 to 0.5 percent by weight, both by reference to the solvent mixture. The temperature for dissolution may be in the range from 30 to 100 and preferably 35 to 70° C. and the drying temperature may be in the range from 30 to 50° C. The precipitate may be washed with an alcohol such as ethanol after isolation, e.g., filtration. The polymorph K can easily be converted in the most stable form B by phase equilibration in e.g., isopropanol and optionally seeding with form B crystals at above room temperature such as temperatures from 30 to 40° C.

#### Hydrate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline hydrate, which shall be described and defined herein as forms C, D, E, H, and O. These hydrate forms are useful as a stable form of BH4 for the pharmaceutical preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

#### Hydrate Form C

It has been found that a hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form C," or "hydrate C." The hydrate form C is slightly hygroscopic and has a water content of approximately 5.5 percent by weight, which indicates that form C is a monohydrate. The hydrate C has a melting point near 94° C. ( $\Delta H_f$  is about 31 J/g) and hydrate form C is especially suitable as intermediate and starting material to produce stable polymorphic forms. Polymorph form C can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form C exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m). FIG. 6 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form C may be obtained by phase equilibration at ambient temperatures of a polymorph form such as polymorph B suspension in a non-solvent, which contains water in an amount of preferably about 5 percent by weight, by reference to the solvent. Hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by suspending (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-solvent such as, heptane, C1-C4-alcohols such as methanol, ethanol, 1- or 2-propanol, acetates, such as ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or binary or ternary mixtures of such non-solvents, to which sufficient water is added to form a monohydrate, and stirring the suspension at or below ambient temperatures (e.g., 0 to 30° C.) for a time sufficient to form a monohydrate. Sufficient water may mean from 1 to 10 and preferably from 3 to 8 percent by weight of water, by reference to the amount of solvent. The solids may be filtered off and dried in air at about room temperature. The solid can absorb some water and therefore possess a higher



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water content than the theoretical value of 5.5 percent by weight. Hydrate form C is unstable with respect to forms D and B, and easily converted to polymorph form B at temperatures of about 40° C. in air and lower relative humidity. Form C can be transformed into the more stable hydrate D by suspension equilibration at room temperature.

Hydrate Form D

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form D," or "hydrate D." The hydrate form D is slightly hygroscopic and may have a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form D is a monohydrate. The hydrate D has a melting point near 153° C. ( $\Delta H_f$  is about 111 J/g) and is of much higher stability than form C and is even stable when exposed to air humidity at ambient temperature. Hydrate form D can therefore either be used to prepare formulations or as intermediate and starting material to produce stable polymorph forms. Polymorph form D can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form D exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w). FIG. 7 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form D may be obtained by adding at about room temperature concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents, and stirring the suspension at ambient temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding at about room temperature a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non-solvent from 1:10 to 1:1000. Form D contains a small excess of water, related to the monohydrate, and it is believed that it is absorbed water due to the slightly hygroscopic nature of this crystalline hydrate. Hydrate form D is deemed to be the most stable one under the known hydrates at ambient temperatures and a relative humidity of less than 70%. Hydrate form D may be used for formulations prepared under conditions, where this hydrate is stable. Ambient temperature may mean 20 to 30° C.

Hydrate Form E

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form E," or "hydrate E." The hydrate form E has a water content of approximately 10 to 14 percent by weight, which suggests that form E is a dihydrate. The hydrate E is formed at temperatures below room temperature. Hydrate

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form E is especially suitable as intermediate and starting material to produce stable polymorph forms. It is especially suitable to produce the water-free form J upon drying under nitrogen or optionally under vacuum. Form E is non-hygroscopic and stable under rather high relative humidities, i.e., at relative humidities above about 60% and up to about 85%. Polymorph form E can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form E exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w). FIG. 8 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form E may be obtained by adding concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent cooled to temperatures from about 10 to -10° C. and preferably between 0 to 10° C. and stirring the suspension at said temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. Hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by adding a concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent, which is cooled to temperatures from about 10 to -10° C., and stirring the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non-solvent from 1:10 to 1:1000. A preferred non-solvent is tetrahydrofuran. Another preparation process comprises exposing polymorph form B to an air atmosphere with a relative humidity of 70 to 90%, preferably about 80%. Hydrate form E is deemed to be a dihydrate, whereby some additional water may be absorbed. Polymorph form E can be transformed into polymorph J upon drying under vacuum at moderate temperatures, which may mean between 20° C. and 50° C. at pressures between 0 and 100 mbar. Form E is especially suitable for formulations in semi solid forms because of its stability at high relative humidities.

Hydrate Form H

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form H," or "hydrate H." The hydrate form H has a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form H is a hygroscopic monohydrate. The hydrate form H is formed at temperatures below room temperature. Hydrate form H is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form H can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Form H exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 8.6 (s), 15.8 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w),

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3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m). FIG. 9 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form H may be obtained by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water, adding then a non-solvent to precipitate a crystalline solid, cooling the obtained suspension and stirring the cooled suspension for a certain time. The crystalline solid is filtered off and then dried under vacuum at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitril, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is tetrahydrofuran. Hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and a less amount than that of acetic acid of water, adding a non-solvent and cooling the obtained suspension to temperatures in the range of -10 to 10° C., and preferably -5 to 5° C., and stirring the suspension at said temperature for a certain time. Certain time may mean 1 to 20 hours. The weight ratio of acetic acid to water may be from 2:1 to 25:1 and preferably 5:1 to 15:1. The weight ratio of acetic acid/water to the non-solvent may be from 1:2 to 1:5. Hydrate form H seems to be a monohydrate with a slight excess of water absorbed due to the hygroscopic nature.

#### Hydrate Form O

It has been found that another hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form O," or "hydrate O." The hydrate form O is formed at temperatures near room temperature. Hydrate form O is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form O can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Form O exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m). FIG. 10 is a graph of the characteristic X-ray diffraction pattern exhibited by hydrate form O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Hydrate form O can be prepared by exposure of polymorphic form F to a nitrogen atmosphere containing water vapor with a resulting relative humidity of about 52% for about 24 hours. The fact that form F, which is a slightly hygroscopic anhydrate, can be used to prepare form O under 52% relative humidity suggests that form O is a hydrate, which is more stable than form F under ambient temperature and humidity conditions.

#### Solvate Forms of (6R) L-Tetrahydrobiopterin Dihydrochloride Salt

As further described below, it has been found that (6R)-L-erythro-tetrahydrobiopterin dihydrochloride exists as a number of crystalline solvate forms, which shall be described and defined herein as forms G, I, L, M, and N. These solvate forms are useful as a stable form of BH4 for the pharmaceutical

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preparations described herein and in the preparation of compositions including stable crystal polymorphs of BH4.

#### Solvate Form G

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form G," or "hydrate G." The ethanol solvate form G has a ethanol content of approximately 8.0 to 12.5 percent by weight, which suggests that form G is a hygroscopic mono ethanol solvate. The solvate form G is formed at temperatures below room temperature. Form G is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form G can be prepared as a solid powder with a desired medium particle size range which is typically ranging from 1 µm to about 500 µm.

Form G exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å) at: 14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w). FIG. 11 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form G may be obtained by crystallization of L-erythro-tetrahydrobiopterin dihydrochloride dissolved in water and adding a large excess of ethanol, stirring the obtained suspension at or below ambient temperatures and drying the isolated solid under air or nitrogen at about room temperature. Here, a large excess of ethanol means a resulting mixture of ethanol and water with less than 10% water, preferably about 3 to 6%. Ethanol form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be prepared by dissolving at about room temperature to temperatures of 75° C. (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water or in a mixture of water and ethanol, cooling a heated solution to room temperature and down to 5 to 10° C., adding optionally ethanol to complete precipitation, stirring the obtained suspension at temperatures of 20 to 5° C., filtering off the white, crystalline solid and drying the solid under air or a protection gas such as nitrogen at temperatures about room temperature. The process may be carried out in a first variant in dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at about room temperature in a lower amount of water and then adding an excess of ethanol and then stirring the obtained suspension for a time sufficient for phase equilibration. In a second variant, (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be suspended in ethanol, optionally adding a lower amount of water, and heating the suspension and dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, cooling down the solution to temperatures of about 5 to 15° C., adding additional ethanol to the suspension and then stirring the obtained suspension for a time sufficient for phase equilibration.

#### Solvate Form I

It has been found that an acetic acid solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form I," or "hydrate I." The acetic acid solvate form I has an acetic acid content of approximately 12.7 percent by weight, which suggests that form I is a hygroscopic acetic acid mono solvate. The solvate form I is formed at temperatures below room temperature. Acetic acid solvate form I is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form I can be

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prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form I exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m), 3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w). FIG. 12 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Acetic acid solvate form I may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water at elevated temperature, adding further acetic acid to the solution, cooling down to a temperature of about 10° C., then warming up the formed suspension to about 15° C., and then stirring the obtained suspension for a time sufficient for phase equilibration, which may last up to 3 days. The crystalline solid is then filtered off and dried under air or a protection gas such as nitrogen at temperatures about room temperature.

Solvate Form L

It has been found that a mixed ethanol solvate/hydrate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form L," or "hydrate L." Form L may contain 4% but up to 13% ethanol and 0% to about 6% of water. Form L may be transformed into form G when treated in ethanol at temperatures from about 0° C. to 20° C. In addition form L may be transformed into form B when treated in an organic solvent at ambient temperatures (10° C. to 60° C.). Polymorph form L can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form L exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w). FIG. 13 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Form L may be obtained by suspending hydrate form E at room temperature in ethanol and stirring the suspension at temperatures from 0 to 10° C., preferably about 5° C., for a time sufficient for phase equilibration, which may be 10 to 20 hours. The crystalline solid is then filtered off and dried preferably under reduced pressure at 30° C. or under nitrogen. Analysis by TG-FTIR suggests that form L may contain variable amounts of ethanol and water, i.e., it can exist as an polymorph (anhydrate), as a mixed ethanol solvate/hydrate, or even as a hydrate.

Solvate Form M

It has been found that an ethanol solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form M," or "hydrate M." Form M may contain 4% but up to 13% ethanol and 0% to about 6% of water, which suggests that form M is a slightly hygroscopic ethanol solvate. The solvate form M is formed at room temperature. Form M is especially suitable as intermediate and starting material to produce stable polymorph forms, since form M can be transformed into form G when treated in ethanol at temperatures between about -10° to 15° C., and into form B when treated in organic solvents such as ethanol, C3 and C4

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alcohols, or cyclic ethers such as THF and dioxane. Polymorph form M can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form M exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs). FIG. 14 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

Ethanol solvate form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in ethanol and evaporation of the solution under nitrogen at ambient temperature, i.e., between 10° C. and 40° C. Form M may also be obtained by drying of form G under a slight flow of dry nitrogen at a rate of about 20 to 100 ml/min. Depending on the extent of drying under nitrogen, the remaining amount of ethanol may be variable, i.e., from about 3% to 13%.

Solvate Form N

It has been found that another solvate crystal form of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is a stable preferred form of BH4 for use in a pharmaceutical preparation described herein, which shall be referred to herein as "form N," "hydrate N." Form N may contain in total up to 10% of isopropanol and water, which suggests that form N is a slightly hygroscopic isopropanol solvate. Form N may be obtained through washing of form D with isopropanol and subsequent drying in vacuum at about 30° C. Form N is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form N can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Form N exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ) at: 19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83 (w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w). FIG. 15 is a graph of the characteristic X-ray diffraction pattern exhibited by solvate form N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride.

The isopropanol form N may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in 4.0 ml of a mixture of isopropanol and water (mixing volume ratio for example 4:1). To this solution is slowly added isopropanol (IPA, for example about 4.0 ml) and the resulting suspension is cooled to 0° C. and stirred for several hours (e.g., about 10 to 18 hours) at this temperature. The suspension is filtered and the solid residue washed with isopropanol at room temperature. The obtained crystalline material is then dried at ambient temperature (e.g., about 20 to 30° C.) and reduced pressure (about 2 to 10 mbar) for several hours (e.g., about 5 to 20 hours). TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water. This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as an non-solvated form containing a small amount of water.

For the preparation of the polymorph forms, there may be used crystallization techniques well known in the art, such as stirring of a suspension (phase equilibration in), precipitation, re-crystallization, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or super-saturated solutions may be used for crystallization, with or without seeding with suitable nucleating agents. Temperatures up to 100° C. may be applied to form solutions. Cooling to initiate crystallization and precipitation down to -100° C. and preferably down to -30° C. may be applied. Meta-stable polymorphs or pseudo-polymorphic forms can



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be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

It was surprisingly found that hydrate form D is the most stable form under the hydrates and forms B and D are especially suitable to be used in pharmaceutical formulations. Forms B and D presents some advantages like an aimed manufacture, good handling due to convenient crystal size and morphology, very good stability under production conditions of various types of formulation, storage stability, higher solubility, and high bioavailability. Accordingly, one embodiment of the compositions and methods disclosed herein is pharmaceutical composition including polymorph form B and/or hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

### III. Pharmaceutical Formulations

The formulations described herein are preferably administered as oral formulations. Oral formulations are preferably solid formulations such as capsules, tablets, pills and troches, or liquid formulations such as aqueous suspensions, elixirs and syrups. The various form of BH4 described herein can be directly used as powder (micronized particles), granules, suspensions or solutions, or it may be combined together with other pharmaceutically acceptable ingredients in admixing the components and optionally finely divide them, and then filling capsules, composed for example from hard or soft gelatin, compressing tablets, pills or troches, or suspend or dissolve them in carriers for suspensions, elixirs and syrups. Coatings may be applied after compression to form pills.

Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be, for example, binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types. The phrase "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that are approved by the U.S. Food and Drug Administration or a corresponding foreign regulatory agency for administration to humans. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compositions, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The initial amount of (6R)-L-erythro-tetrahydrobiopterin used to prepare the formulation may be, for example, in the range of about 30 wt % to about 40 wt % of the formulation, or in the range of about 32 wt % to about 35 wt %, or at about 33 wt %. Specific amounts of BH4 in a formulation contemplated herein include 80 mg, 100 mg, 200 mg, 300 mg, 400 mg, and 500 mg.

Binders assist in maintaining a solid formulation. In some cases, anhydrous binders are used to preserve the anhydrous state of polymorph forms. In some cases, the binder may act as a drying agent. Exemplary binders include anhydrous dibasic calcium phosphate and its monohydrate. Other nonlimiting examples of binders useful in a composition described herein include gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or

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co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-d,l-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (poly-oxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol and esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidone, and natural polymers like chitosan.

Disintegration agents assist in rapid disintegration of solid formulations by absorbing water and expanding. Exemplary disintegration agents include polyvinylpyrrolidone (PVP, e.g. sold under the name POVIDONE), a cross-linked form of povidone (CPVP, e.g. sold under the name CROSPOVIDONE), a cross-linked form of sodium carboxymethylcellulose (NaCMC, e.g. sold under the name AC-DI-SOL), other modified celluloses, and modified starch. Tablets formulated with CPVP exhibited much more rapid disintegration than tablets formulated with PVP.

Antioxidants may be included and help stabilize the tetrahydrobiopterin product, especially after dissolution. Low pH aqueous solutions of API are more stable than are solutions at neutral or high pH. Antioxidants are included in a formulation described herein to prevent deterioration from oxidation. Antioxidants can generally be classified into 3 groups.

The first group is known as true antioxidants, and inhibit oxidation by reacting with free radicals blocking the chain reaction. Examples include phenolic antioxidants, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl-hydroquinone (TBHQ), 4-hydroxymethyl-2,6-di-tert-butylphenol (HMBP), and 2,4,5-trihydroxybutyrophenone (THBP); alkylgallates, including propyl gallate; gallic acid; nordihydroguaiaretic acid; and tocopherols, including alpha-tocopherol.

The second group, consisting of reducing agents, have lower redox potentials than the drug which they are intended to protect, and are therefore more readily oxidized. Reducing agents may act also by reacting with free radicals. Examples include ascorbic acid, thioglycolic acid (TGA), ascorbyl palmitate, sulfites, including potassium and sodium salts of sulphurous acid (e.g., potassium sulfite, sodium sulfite, sodium metabisulphite, and sodium bisulfite), and thioglycerol.

The third group consists of antioxidant synergists which usually have a modest antioxidant effect themselves but probably enhance the action of antioxidants in the first or second group by reacting with heavy metal ions which catalyze oxidation. Examples of such antioxidant synergists and chelating agents include citric acid, malic acid, editic acid and its salts, lecithin, and tartaric acid.

Exemplary acidic antioxidants include ascorbic acid, fatty acid esters of ascorbic acid such as ascorbyl palmitate and ascorbyl stearate, and salts of ascorbic acid such as sodium, calcium, or potassium ascorbate. Non-acidic antioxidants may also be used in the stable tablet formulations. Nonlimiting examples of non-acidic antioxidants include beta-carotene, alpha-tocopherol. Acidic additives may be added to enhance stability of the tablet formulation, including citric acid or malic acid. Small molecule anti-oxidants include but are not limited to thiols, e.g., cysteine, N-acetyl cysteine, glutathione, etc., or thiolated polymers (polymer-SH), e.g.,

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polycarboxophil-cysteine, polymethacrylic-SH, carboxy methylcellulose-cysteine, etc. or small molecule anti-oxidants such as ascorbic acid, methionine, ascorbyl palmitate, etc. These anti-oxidants confer stability on the dosage form during transit through the GIT, particularly as the pH of the GIT increases with distance from the stomach.

In one embodiment, a combination of at least two reducing agent antioxidants is preferred. In another embodiment, a combination of at least two reducing agent antioxidants together with an acid antioxidant synergist and/or chelating agent is preferred.

Lubricants improve stability, hardness and uniformity of solid formulations. Exemplary lubricants include stearyl fumarate and magnesium stearate. Other nonlimiting examples of lubricants include natural or synthetic oils, fats, waxes, or fatty acid salts such as magnesium stearate.

Optionally the stable formulations of the invention can also comprise other excipients such as mannitol, hydroxyl propyl cellulose, microcrystalline cellulose, or other non-reducing sugars such as sucrose, trehalose, melezitose, planteose, and raffinose. Reducing sugars may react with BH4. Other non-limiting examples of excipients useful in a composition described herein include phosphates such as dicalcium phosphate.

Surfactants for use in a composition described herein can be anionic, anionic, amphoteric or neutral. Nonlimiting examples of surfactants useful in a composition described herein include lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1 -acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexadecanoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium dioctylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Nonlimiting examples of sweetening agents useful in a composition described herein include sucrose, fructose, lactose or aspartame. Nonlimiting examples of flavoring agents for use in a composition described herein include peppermint, oil of wintergreen or fruit flavors such as cherry or orange flavor. Nonlimiting examples of coating materials for use in a composition described herein include gelatin, wax, shellac, sugar or other biological degradable polymers. Nonlimiting examples of preservatives for use in a composition described herein include methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

The BH4 form may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution. Slow release formulations may also be prepared in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers.

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Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

The BH4 used in a composition described herein is preferably formulated as a dihydrochloride salt, however, it is contemplated that other salt forms of BH4 possess the desired biological activity, and consequently, other salt forms of BH4 can be used. Specifically, BH4 salts with inorganic or organic acids are preferred. Nonlimiting examples of alternative BH4 salts forms includes BH4 salts of acetic acid, citric acid, oxalic acid, tartaric acid, fumaric acid, and mandelic acid.

Pharmaceutically acceptable base addition salts may be formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. Examples of metals used as cations are sodium, potassium, magnesium, ammonium, calcium, or ferric, and the like. Examples of suitable amines include isopropylamine, trimethylamine, histidine, N,N' dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N methylglucamine, and procaine.

Pharmaceutically acceptable acid addition salts include inorganic or organic acid salts. Examples of suitable acid salts include the hydrochlorides, acetates, citrates, salicylates, nitrates, phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include, for example, acetic, citric, oxalic, tartaric, or mandelic acids, hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4 aminosalicic acid, 2 phenoxybenzoic acid, 2 acetoxymbenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2 hydroxyethanesulfonic acid, ethane 1,2 disulfonic acid, benzenesulfonic acid, 4 methylbenzenesulfonic acid, naphthalene 2 sulfonic acid, naphthalene 1,5 disulfonic acid, 2 or 3 phosphoglycerate, glucose 6 phosphate, N cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid.

Exemplary stable oral formulations contain one or more of the following additional ingredients that improve the stability or other characteristics of the formulation: binder, disintegration agent, acidic antioxidant, or lubricant or combinations thereof. Exemplary stable tablet formulations include a binder and disintegration agent, optionally with an acidic antioxidant, and optionally further including a lubricant. Exemplary concentrations of binder are between about 1 wt % to about 5 wt %, or between about 1.5 and 3 wt %; an exemplary weight ratio of binder to BH4 is in the range of about 1:10 to about 1:20. Exemplary concentrations of disintegration agent are between about 1 wt % to about 20 wt %; an exemplary weight ratio of disintegration agent to BH4 is in the range of about 1:5 to about 1:10. Exemplary concentra-

tions of antioxidant are between about 1 wt % and about 3 wt %; an exemplary weight ratio of antioxidant to BH4 is in the range of about 1:5 to 1:30. In one example, ascorbic acid is the antioxidant and is used at a ratio to BH4 of less than 1:1, e.g. 1:2 or less, or 1:10 or less. Exemplary concentrations of lubricant in a stable tablet formulation of the present invention are between about 0.1 wt % and about 2 wt %; an exemplary weight ratio of lubricant to BH4 is in the range of about 1:25 to 1:65.

The stable solid formulation may optionally include other therapeutic agents suitable for the condition to be treated, e.g. folates, including folate precursors, folic acids, or folate derivatives; and/or arginine; and/or vitamins, such as vitamin C and/or vitamin B2 (riboflavin) and/or vitamin B12; and/or neurotransmitter precursors such as L-dopa or carbidopa; and/or 5-hydroxytryptophan.

Exemplary folates, including folate precursors, folic acids, or folate derivatives, are disclosed in U.S. Pat. Nos. 6,011,040 and 6,544,994, both of which are incorporated herein by reference, and include folic acid (pteroylmonoglutamate), dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-methylenetetrahydrofolic acid, 5,10-formiminotetrahydrofolic acid, 5-formyltetrahydrofolic acid (leucovorin), 10-formyltetrahydrofolic acid, 10-methyltetrahydrofolic acid, one or more of the folypolyglutamates, compounds in which the pyrazine ring of the pterin moiety of folic acid or of the folypolyglutamates is reduced to give dihydrofolates or tetrahydrofolates, or derivatives of all the preceding compounds in which the N-5 or N-10 positions carry one carbon units at various levels of oxidation, or pharmaceutically compatible salts thereof, or a combination of two or more thereof. Exemplary tetrahydrofolates include 5-formyl-(6S)-tetrahydrofolic acid, 5-methyl-(6S)-tetrahydrofolic acid, 5,10-methylene-(6R)-tetrahydrofolic acid, 5,10-methenyl-(6R)-tetrahydrofolic acid, 10-formyl-(6R)-tetrahydrofolic acid, 5-formimino-(6S)-tetrahydrofolic acid or (6S)-tetrahydrofolic acid, and pharmaceutically acceptable salts thereof. Exemplary salts include sodium, potassium, calcium or ammonium salts.

Exemplary relative weight ratios of BH4 to folates to arginine may be from about 1:10:10 to about 10:1:1.

The stable formulations of the invention may be provided, e.g. as tablets or pills or capsules in HDPE bottles provided with a dessicant capsule or pouch; or in foil-on-foil blister packaging, or in blister packaging comprising see-through polymer film, if commercially desirable.

#### IV. Treatment Of Bh4-Responsive Diseases

Hyperphenylalaninemia, Neuropsychological or Neuropsychiatric Disorders

The methods of the invention may be used for treatment of conditions associated with elevated phenylalanine levels or decreased tyrosine or tryptophan levels, which may be caused, for example, by reduced phenylalanine hydroxylase, tyrosine hydroxylase, or tryptophan hydroxylase activity. Conditions associated with elevated phenylalanine levels specifically include phenylketonuria, both mild and classic, and hyperphenylalaninemia as described herein, and exemplary patient populations include the patient subgroups described herein as well as any other patient exhibiting phenylalanine levels above normal.

Conditions associated with decreased tyrosine or tryptophan levels include neurotransmitter deficiency, neurological and psychiatric disorders such as Parkinson's, dystonia, spinocerebellar degeneration, pain, fatigue, depression, other affective disorders and schizophrenia. NO overproduction by nNOS has been implicated in strokes, migraine headaches,

Alzheimer's disease, and with tolerance to and dependence on morphine. BH4 may be administered for any of these conditions. Other exemplary neuropsychiatric disorders for which BH4 may be administered include Parkinson's disease, Alzheimer's disease, schizophrenia, schizophreniform disorder, schizoaffective disorder, brief psychotic disorder, delusional disorder, shared psychotic disorder, psychotic disorder due to a general medical condition, substance-induced psychotic disorder, other psychotic disorders, tardive dyskinesia, Machado-Joseph disease, spinocerebellar degeneration, cerebellar ataxia, dystonia, chronic fatigue syndrome, acute or chronic depression, chronic stress syndrome, fibromyalgia, migraine, attention deficit hyperactivity disorder, bipolar disease, and autism.

The stable formulations may also be used for treating patients suffering from BH4 deficiency, e.g., due to a defect in the pathway for its synthesis, including but not limited to dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, or dihydropteridine reductase (DHPR) deficiency.

Suitable subjects for treatment with the stable formulations of the invention include subjects with an elevated plasma Phe concentration in the absence of the therapeutic, e.g. greater than 1800  $\mu\text{M/L}$ , or greater than 1600  $\mu\text{M}$ , greater than 1400  $\mu\text{M}$ , greater than 1200  $\mu\text{M}$ , greater than 1000  $\mu\text{M}$ , greater than 800  $\mu\text{M}$ , or greater than 600  $\mu\text{M}$ , greater than 420  $\mu\text{M}$ , greater than 300  $\mu\text{M}$ , greater than 200  $\mu\text{M}$ , or greater than 180  $\mu\text{M}$ . Mild PKU is generally classified as plasma Phe concentrations of up to 600  $\mu\text{M/L}$ , moderate PKU as plasma Phe concentrations of between 600  $\mu\text{M/L}$  to about 1200  $\mu\text{M/L}$  and classic or severe PKU as plasma Phe concentrations that are greater than 1200  $\mu\text{M/L}$ . Preferably treatment with the stable formulations alone or with protein-restricted diet decreases the plasma phenylalanine concentration of the subject to less than 600  $\mu\text{M}$ , or less than 500  $\mu\text{M}$ , or 360  $\mu\text{M} \pm 15 \mu\text{M}$  or less, or less than 200  $\mu\text{M}$ , or less than 100  $\mu\text{M}$ . Other suitable subjects include subjects diagnosed as having a reduced phenylalanine hydroxylase (PAH) activity, atypical or malignant phenylketonuria associated with BH4 deficiency, hyperphenylalaninemia associated with liver disorder, and hyperphenylalaninemia associated with malaria. Reduced PAH activity may result from a mutation in the PAH enzyme, for example, a mutation in the catalytic domain of PAH or one or more mutations selected from the group consisting of F39L, L48S, I65T, R68S, A104D, S110C, D129G, E178G, V190A, P211T, R241C, R261Q, A300S, L308F, A313T, K320N, A373T, V388M E390G, A395P, P407S, and Y414C; or subjects that are pregnant females, females of child-bearing age that are contemplating pregnancy, or infants between 0 and 3 years of age, or 0-2, 0-1.5 or 0-1; or subjects diagnosed as unresponsive within 24 hours to a single-dose BH4 loading test or a multiple dose loading test, such as a 4-dose or 7-day loading test. Exemplary patient populations and exemplary BH4 loading tests are described in Int'l. Publication No. WO 2005/049000, incorporated herein by reference in its entirety. U.S. Pat. Nos. 4,752,573; 4,758,571; 4,774,244; 4,920,122; 5,753,656; 5,922,713; 5,874,433; 5,945,452; 6,274,581; 6,410,535; 6,441,038; 6,544,994; and U.S. Patent Publications US 20020187958; US 20020106645; US 2002/0076782; US 20030032616 (each incorporated herein by reference) each describe methods of administering BH4 compositions for non-PKU treatments. Each of those patents is incorporated herein by reference as providing a general teaching of methods of administering BH4 compositions known to those of skill in the art, that may be adapted for the treatment as described herein.

While individual needs vary, determination of optimal ranges of effective amounts of each component is within the



skill of the art. Typical dosages of the BH4 comprise about 1 to about 20 mg/kg body weight per day, which will usually amount to about 5 (1 mg/kg×5 kg body weight) to 3000 mg/day (30 mg/kg×100 kg body weight). While continuous, daily administration is contemplated, for HPA it may be desirable to cease the BH4 therapy when the symptoms of Phe levels are reduced to below a certain threshold level. Of course, the therapy may be reinitiated in the event that Phe levels rise again. Appropriate dosages may be ascertained through the use of established assays for determining blood levels of Phe in conjunction with relevant dose response data.

In exemplary embodiments, it is contemplated that the methods of the present invention will provide to a patient in need thereof, a daily dose of between about 10 mg/kg to about 20 mg/kg of BH4. Of course, one skilled in the art may adjust this dose up or down depending on the efficacy being achieved by the administration. The daily dose may be administered in a single dose or alternatively may be administered in multiple doses at conveniently spaced intervals. In exemplary embodiments, the daily dose may be 1 mg/kg, 2 mg/kg, 3 mg/kg, 4 mg/kg, 5 mg/kg, 6 mg/kg, 7 mg/kg, 8 mg/kg, 9 mg/kg, 10 mg/kg, 11 mg/kg, 12 mg/kg, 13 mg/kg, 14 mg/kg, 15 mg/kg, 16 mg/kg, 17 mg/kg, 18 mg/kg, 19 mg/kg, 20 mg/kg, 22 mg/kg, 24 mg/kg, 26 mg/kg, 28 mg/kg, 30 mg/kg, 32 mg/kg, 34 mg/kg, 36 mg/kg, 38 mg/kg, 40 mg/kg, 42 mg/kg, 44 mg/kg, 46 mg/kg, 48 mg/kg, 50 mg/kg, or more mg/kg.

#### Low Dose Regimens

In a low dose therapeutic method of the invention, low doses, e.g., doses of 0.1 to 5 mg/kg per day are contemplated, including doses of 0.1 to 2 mg/kg, or 0.1 to 3 mg/kg, or 1 mg/kg to 5 mg/kg. Doses of less than 5 mg/kg per day are preferred. According to the invention, such doses are expected to provide improvements with relevant study endpoints, and BH4 derivatives are expected to have improved biological properties relative to natural BH4 at such doses. In particular, the invention contemplates that any of the 1',2'-diacyl-(6R,S)-5,6,7,8-tetrahydro-L-biopterins or lipoidal tetrahydrobiopterins described herein exhibit improved biological properties at low doses.

The invention also specifically contemplates the use of BH4, or a precursor or derivative thereof, for treating BH4-responsive diseases at a dose in the range of 0.1 to 5 mg/kg body weight/day, via any route of administration including but not limited to oral administration, in a once daily dose or multiple (e.g. 2, 3 or 4) divided doses per day, for a duration of at least 1, 2, 3, or 4 weeks or longer, or 1, 2, 3, 4, 5, 6 months or longer. Exemplary doses include less than 5 mg/kg/day, 4.5 mg/kg/day or less, 4 mg/kg/day or less, 3.5 mg/kg/day or less, 3 mg/kg/day or less, 2.5 mg/kg/day or less, 2 mg/kg/day or less, 1.5 mg/kg/day or less, 1 mg/kg/day or less, or 0.5 mg/kg/day or less. Equivalent doses per body surface area are also contemplated.

For the person of average weight/body surface area (e.g. 70 kg), the invention also contemplates a total daily dose of less than 400 mg. Exemplary such total daily doses include 360 mg/day, 350 mg/day, 300 mg/day, 280 mg/day, 210 mg/day, 180 mg/day, 175 mg/day, 150 mg/day, or 140 mg/day. For example, 350 mg/day or 175 mg/day is easily administrable with an oral dosage formulation of 175 mg, once or twice a day. Other exemplary total daily doses include 320 mg/day or less, 160 mg/day or less, or 80 mg/day or less. Such doses are easily administrable with an oral dosage formulation of 80 or 160 mg. Other exemplary total daily doses include 45, 90, 135, 180, 225, 270, 315 or 360 mg/day or less, easily administrable with an oral dosage formulation of 45 or 90 mg. Yet other exemplary total daily doses include 60, 120, 180, 240,

300, or 360 mg/day, easily administrable with an oral dosage formulation of 60 or 120 mg. Other exemplary total daily doses include 70, 140, 210, 280, or 350 mg/day, easily administrable with an oral dosage formulation of 70 or 140 mg. Exemplary total daily doses also include 55, 110, 165, 220, 275 or 330 mg/day, easily administrable with an oral dosage formulation of 55 mg. Other exemplary total daily doses include 65, 130, 195, 260, or 325 mg/day, or 75, 150, 225, 300 or 375 mg/day, e.g. in dosage formulations of 65 mg or 75 mg. Diseases Associated with Nitric Oxide Synthase Dysfunction

The invention further contemplates that stable formulations of the invention may be used for treatment of subjects suffering from conditions that would benefit from enhancement of nitric oxide synthase activity and patients suffering from vascular diseases, ischemic or inflammatory diseases, or insulin resistance. The treatment may, for example alleviate a deficiency in nitric oxide synthase activity or may, for example provide an increase in nitric oxide synthase activity over normal levels. It has been suggested that a patient suffering from a deficiency in nitric oxide synthase activity would benefit from co-treatment with folates, including folate precursors, folic acids, or folate derivatives.

Nitric oxide is constitutively produced by vascular endothelial cells where it plays a key physiological role in the regulation of blood pressure and vascular tone. It has been suggested that a deficiency in nitric oxide bioactivity is involved in the pathogenesis of vascular dysfunctions, including coronary artery disease, atherosclerosis of any arteries, including coronary, carotid, cerebral, or peripheral vascular arteries, ischemia-reperfusion injury, hypertension, diabetes, diabetic vasculopathy, cardiovascular disease, peripheral vascular disease, or neurodegenerative conditions stemming from ischemia and/or inflammation, such as stroke, and that such pathogenesis includes damaged endothelium, insufficient oxygen flow to organs and tissues, elevated systemic vascular resistance (high blood pressure), vascular smooth muscle proliferation, progression of vascular stenosis (narrowing) and inflammation. Thus, treatment of any of these conditions is contemplated according to methods of the invention.

It has also been suggested that the enhancement of nitric oxide synthase activity also results in reduction of elevated superoxide levels, increased insulin sensitivity, and reduction in vascular dysfunction associated with insulin resistance, as described in U.S. Pat. No. 6,410,535, incorporated herein by reference. Thus, treatment of diabetes (type I or type II), hyperinsulinemia, or insulin resistance is contemplated according to the invention. Diseases or disorders having vascular dysfunction associated with insulin resistance include those caused by insulin resistance or aggravated by insulin resistance, or those for which cure is retarded by insulin resistance, include but are not limited to abnormal vascular compliance, endothelial dysfunction and hypertension, disorders of insulin sensitivity and glucose control, abnormal peripheral perfusion such as intermittent claudication, reduced peripheral perfusion, decreased skin blood flow, defective wound healing and peripheral circulation disorder, hyperlipidemia, arteriosclerosis, coronary vasoconstrictive angina, effort angina, cerebrovascular constrictive lesion, cerebrovascular insufficiency, cerebral vasospasm, coronary arteriostenosis following percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG), obesity, insulin-independent diabetes, hyperinsulinemia, lipid metabolism abnormality, coronary arteriosclerotic heart diseases, congestive heart failure, pulmonary hypertension with or without congestive heart failure, exercise-associated angina, coronary artery disease and related

atherosclerosis; ophthalmic disease such as optic atrophy and diabetic retinal disease; and renal disease such as microalbuminuria in diabetic renal disease, renal failure and decreased glomerular filtration rate.

It is contemplated that when administered to patients with these diseases, BH4 can prevent or treat these diseases by activating the functions of NOS, increasing NO production and suppressing the production of active oxygen species to improve disorders of vascular endothelial cells.

The invention provides a method for treating a subject diagnosed as having vascular disease unrelated to diabetes selected from the group consisting of pulmonary vascular disease, hemolytic anemias, stroke and related ischemic vascular disease (such as stroke, cardiac or coronary disease, arteriosclerosis, or peripheral vascular disease), thrombosis, transplant-related endothelial dysfunction, and cardiac or coronary disease. In one embodiment, pulmonary vascular disease includes but is not limited to pulmonary tension in sickle cell anemia and other hemoglobinopathies, idiopathic pulmonary hypertension, persistent pulmonary hypertension of the newborn (PPHN). In a further embodiment, hemolytic anemias include hereditary hemolytic anemias and acquired hemolytic anemia. Hereditary hemolytic anemias include but are not limited to sickle-cell anemia, thalassemia, hemolytic anemia due to G6PD deficiency, pyruvate kinase deficiency, hereditary elliptocytosis, hereditary spherocytosis, hereditary stomatocytosis, hereditary ovalocytosis, paroxysmal nocturnal hemoglobinuria, and hemoglobin SC disease. Acquired hemolytic anemias include but are not limited to microangiopathic hemolytic anemia, idiopathic autoimmune hemolytic anemia, non-immune hemolytic anemia caused by chemical or physical agents or devices (left ventricular assist devices), mechanical heart valves and bypass devices), and secondary immune hemolytic anemia.

In another embodiment, stroke and related ischemic vascular disease includes but is not limited to vasospasm, such as post-stroke cerebrovascular spasm. Thrombosis includes but is not limited to thrombogenesis, thrombosis, clotting, and coagulation. In a further embodiment, transplant-related endothelial dysfunction includes but is not limited to vascular dysfunction after solid organ transplantation and cyclosporine A induced endothelial dysfunction. In yet another embodiment, cardiac or coronary disease includes but is not limited to congestive heart failure, vascular dysfunction and angina associated with hypercholesterolemia, and vascular dysfunction and angina associated with tobacco smoking.

BH4 can also prevent or treat other disorders associated with the overproduction of or damage related to reactive oxygen species, including but not limited to sepsis.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired (i.e., the amount of decrease in plasma Phe concentration desired). The frequency of dosing also is dependent on pharmacodynamic effects on Phe levels. If the effect lasts for 24 hours from a single dose. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

The frequency of BH4 dosing will depend on the pharmacokinetic parameters of the agent and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. See for example Rem-

ington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publ. Co, Easton Pa. 18042) pp 1435 1712, incorporated herein by reference. Such formulations may influence the physical state, stability, rate of in vivo release and rate of in vivo clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface areas or organ size. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

#### V. Combination Therapy

Certain methods of the invention involve the combined use of the stable formulations of the invention and one or more other therapeutic agents.

In such combination therapy, administration of the stable formulations of the invention may be concurrent with or may precede or follow the administration of the second therapeutic agent, e.g. by intervals ranging from minutes to hours, so long as both agents are able to exert their therapeutic effect at overlapping time periods. Thus, the invention contemplates the stable formulations of the invention for use with a second therapeutic agent.

The invention also contemplates use of a second therapeutic agent in preparation of a medicament for administration with the stable tetrahydrobiopterin, precursor, derivative or analog formulations of the invention.

Tetrahydrobiopterin therapy may be combined with dietary protein restriction to effect a therapeutic outcome in patients with various forms of HPA. For example, one could administer to the subject the BH4 composition and a low-phenylalanine medical protein composition in a combined amount effective to produce the desired therapeutic outcome (i.e., a lowering of plasma Phe concentration and/or the ability to tolerate greater amounts of Phe/protein intake without producing a concomitant increase in plasma Phe concentrations). This process may involve administering the BH4 composition and the dietary protein therapeutic composition at the same time. This may be achieved by administering a single composition or pharmacological protein formulation that includes all of the dietary protein requirements and also includes the BH4 within said protein formulation. Alternatively, the dietary protein (supplement or normal protein meal) is taken at about the same time as a pharmacological formulation (tablet, injection or drink) of BH4.

In some embodiments, the protein-restricted diet is one which is supplemented with amino acids, such as tyrosine, valine, isoleucine and leucine. The patient may be co-administered a low-Phe protein supplement, which may include L-tyrosine, L-glutamine, L-carnitine at a concentration of 20 mg/100 g supplement, L-taurine at a concentration of 40 mg/100 g supplement and selenium. It may further comprise the recommended daily doses of minerals, e.g., calcium, phosphorus and magnesium. The supplement further may comprise the recommended daily dose of one or more amino acids selected from the group consisting of L-leucine, L-pro-

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line, L-lysine acetate, L-valine, L-isoleucine, L-arginine, L-alanine, glycine, L-asparagine monohydrate, L-tryptophan, L-serine, L-threonine, L-histidine, L-methionine, L-glutamic acid, and L-aspartic acid. In addition, the supplement may be fortified with the recommended daily dosage of vitamins A, D and E. Optionally, the supplement comprises a fat content that provides at least 40% of the energy of the supplement. Such supplements may be provided in the form of a powder supplement or in the form of a protein bar. In certain embodiments, protein-restricted diet comprises a protein supplement and the BH4 is provided in the same composition as the protein supplement.

In other alternatives, the BH4 treatment may precede or follow the dietary protein therapy by intervals ranging from minutes to hours. In embodiments where the protein and the BH4 compositions are administered separately, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the BH4 will still be able to exert an advantageously effect on the patient. In such instances, it is contemplated that one would administer the BH4 within about 2-6 hours (before or after) of the dietary protein intake, for example, with a delay time of only about 1 hour or less. In certain embodiments, it is contemplated that the BH4 therapy will be a continuous therapy where a daily dose of BH4 is administered to the patient indefinitely. In other situations, e.g., in pregnant women having only the milder forms of PKU and HPA, it may be that the BH4 therapy is only continued for as long as the woman is pregnant and/or breast feeding.

Further, in addition to therapies based solely on the delivery of BH4 and dietary protein regulation, the methods of the present invention also contemplate combination therapy with a third composition that specifically targets one or more of the symptoms of HPA. For example, it is known that the deficit in tyrosine caused by HPA results in a deficiency in neurotransmitters dopamine and serotonin. Thus, in the context of the present invention, it is contemplated that BH4 and dietary protein based methods could be further combined with administration of L-dopa, carbidopa and 5-hydroxytryptophan neurotransmitters to correct the defects that result from decreased amounts of tyrosine in the diet.

In addition, gene therapy with both PAH (Christensen et al., *Mol. Gent. And Metabol.* 76: 313-318, 2002; Christensen et al., *Gene Therapy*, 7:1971-1978, 2000) and phenylalanine ammonia-lyase (PAL Liu et al., *Arts. Cells. Blood. Subs and Immob. Biotech.* 30(4)243-257, 2002) has been contemplated by those of skill in the art. Such gene therapy techniques could be used in combination with the combined BH4/dietary protein restriction based therapies of the invention. In further combination therapies, it is contemplated that phenylase may be provided as an injectable enzyme to destroy lower Phe concentrations in the patient. As the administration of phenylase would not generate tyrosine (unlike administration of PAH), such treatment will still result in tyrosine being an essential amino acid for such patients. Therefore dietary supplementation with tyrosine may be desirable for patients receiving phenylase in combination with the BH4 therapy.

BH4 may be co-administered for neuropsychological or neuropsychiatric disorders according to the method of the invention with one or more other neuropsychiatric active agents, including antidepressants, neurotransmitter precursors such as tryptophan, tyrosine, serotonin, agents which activate noradrenergic systems, such as lofepramine, desipramine, reboxetine, tyrosine, agents which act preferentially on serotonin, combined inhibitors of both noradrenaline and serotonin uptake, such as venlafaxine, duloxetine or mil-

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nacipran, or drugs which are combined inhibitors of both dopamine and noradrenaline reuptake such as bupropion.

In a related embodiment, BH4 is administered with other therapeutic agents commonly used to treat diabetes, vascular disease, hyperlipidemia. Agents used to treat diabetes, include but not limited to agents that improve insulin sensitivity such as PPAR gamma ligands (thiazolidinediones, glitazones, troglitazones, rosiglitazone (Avandia), pioglitazone), stimulators of insulin secretion such as sulphonylureas (gliquidone, tolbutamide, glimepiride, chlorpropamide, glipizide, glyburide, acetohexamide) and meglitinides (meglitinide, repaglinide, nateglinide) and agents that reduce liver production of glucose such as metformin. Agent used to treat vascular disease, include but not limited to endothelin receptor antagonists commonly used for the treatment of hypertension and other endothelial dysfunction-related disorders, such as bosentan, darusentan, enrasentan, tezosestan, atrasentan, ambrisentan sitaxsentan; smooth muscle relaxants such as PDE5 inhibitors (indirect-acting) and minoxidil (direct-acting); angiotensin converting enzyme (ACE) inhibitors such as captopril, enalapril, lisinopril, fosinopril, perindopril, quinapril, trandolapril, benazepril, ramipril; angiotensin II receptor blockers such as irbesartan, losartan, valsartan, eprosartan, olmesartan, candesartan, telmisartan; beta blockers such as atenolol, metoprolol, nadolol, bisoprolol, pindolol, acebutolol, betaxolol, propranolol; diuretics such as hydrochlorothiazide, furosemide, torsemide, metolazone; calcium channel blockers such as amlodipine, felodipine, nisoldipine, nifedipine, verapamil, diltiazem; alpha receptor blockers doxazosin, terazosin, alfuzosin, tamsulosin; and central alpha agonists such as clonidine. Agents used to treat hyperlipidemia, include but not limited to agents that lower LDL such as statins (atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin calcium, simvastatin) and nicotinic acid, cholesteryl ester transfer protein inhibitors (such as torcetrapib), agents that stimulate PPAR alpha such as fibrates, gemfibrozil, fenofibrate, bezafibrate, ciprofibrate, agents that bind and prevent reabsorption of bile acids and reduce cholesterol levels such as bile acid sequestrants, cholestyramine and colestipol, and cholesterol absorption inhibitors.

BH4 may also be administered with a factor or combination of factors that enhances or normalizes the production of the vasodilator nitric oxide (NO) alone or in combination with a therapeutic agent. In one embodiment, such factor(s) enhances the activity or expression the de novo biosynthesis of BH4 and is selected from the group consisting of guanosine triphosphate cyclohydrolase I (GTPCH1), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase. In a preferred embodiment of the invention, BH4 synthesis is increased by increasing the expression of GTPCH1 expression by the use of any one or more cyclic adenosine monophosphate (cAMP) analogs or agonists including forskolin, 8-bromo cAMP or other agents that function to increase cAMP mediated cell signaling, for example, cytokines and growth factors including interleukin-1, interferon-gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), c-reactive protein, HMG-CoA-reductases (statins like atorvastatin) nerve growth factor (NGF), epidermal growth factor (EGF), hormones including adrenomedullin and estradiol benzoate, and other compounds such as NADPH and NADPH analogs, caffeine, cyclosporine A methyl-xanthines including 3-isobutyl-1-methyl xanthine, theophylline, reserpine, hydrogen peroxide.

One embodiment of invention therefore relates to increasing GTPCH1 levels by inhibiting the degradation of 3'5'-cyclic nucleotides using inhibitors of the eleven phosphodi-



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esterases families (PDE1-11) including PDE1, PDE3, PDE5. The PDE inhibitors of the present invention include Viagra/sildenafil, cialis/tadalafil, vardenafil/levitra, udenafil, 8-Methoxymethyl-IBMX, UK-90234, dexamethasone, hesperetin, hesperidins, Irsogladine, vinpocetine, cilostamide, rolipram, ethyl beta-carboline-3-carboxylate (beta-CCE), tetrahydro-beta-carboline derivatives, 3-O-methylquercetin and the like.

Another embodiment of the invention relates to increasing the levels of BH4 by increasing the levels of BH4-synthesizing enzymes by gene therapy or endothelium-targeted delivery of polynucleotides of the synthetic machinery of BH4. Yet another embodiment of the invention relates to increasing the levels of BH4 by supplementation with BH4-synthesizing enzymes GTPCH1, PTPS, SR, PCD, DHPR and DHFR. It is contemplated that BH4-synthesizing enzymes encompasses all natural and unnatural forms of the enzymes including mutants of the proteins.

Another embodiment of the invention relates to increasing BH4 levels by diverting the substrate 7,8-dihydroneopterin triphosphate towards BH4 synthesizing enzyme PTPS instead of alkaline phosphatase (AP) by inhibiting AP activity. The agents or compounds that inhibit the activity of AP include phosphate analogs, levamisole, and L-Phe. Another embodiment of the invention relates to agents or compounds that inhibit alkaline phosphatase includes the small inhibitory RNA (siRNA), antisense RNA, dsDNA, small molecules, neutralizing antibodies, single chain, chimeric, humanized and antibody fragments to inhibit the synthesis of alkaline phosphatase.

Another embodiment of the invention includes agents or compounds that enhance the activity of catalysts or cofactors needed for the synthesis of enzymes of the de novo synthesis pathway of BH4 synthesis.

Another embodiment of the invention includes agents or compounds that prevent the degradation of the enzymes needed for the synthesis of BH4. Yet another embodiment of the invention includes agents or compounds that prevent the degradation of the catalysts needed for the synthesis of BH4 and its synthetic enzymes including GTPCH1, PTPS and SR.

Another embodiment of the invention relates to increasing the levels of BH4 by increasing the reduction of BH2 via the salvage pathway. In vivo, BH4 becomes oxidized to BH2. BH2 which exist as the quinoid form (qBH2) and as the 7,8-dihydropterin which is reduced to BH4 by DHPR and DHFR respectively. One embodiment of the invention relates to increasing the regeneration or salvage of BH4 from BH2 by modulating the activity and synthesis of the enzymes PCD, DHPR and DHFR using agents or compounds that pathway NADPH, thiols, perchloromercuribenzoate, hydrogen peroxide and the like.

Another embodiment of the invention relates to agents that stabilize BH4 by decreasing the oxidation of BH4 using agents or compounds such as antioxidants including ascorbic acid (vitamin C), alpha tocopherol (vitamin E), tocopherols (e.g vitamin A), selenium, beta-carotenes, carotenoids, flavones, flavonoids, folates, flavones, flavanones, isoflavones, catechins, anthocyanidins, and chalcones.

In a further embodiment, such factor(s) may increase the activity or expression of nitric oxide synthase and thereby enhance the generation of NO.

In yet another embodiment, the invention contemplates factors that inhibit the GTPCH feedback regulatory protein, GFRP. An embodiment of the invention relates to agents or compounds that inhibit the binding of BH4 to the GTPCH1/GFRP complex, thereby preventing the feedback inhibition by BH4. Agents or compounds of this invention include com-

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petitive inhibitors such as alternate forms of BH4 with altered affinities for the complex, structural analogs etc. Still another embodiment of the invention includes agents or compounds that enhance the binding of L-phenylalanine to CTPCH1/GFRP inducing the synthesis of BH4. Another embodiment of the invention includes agents or compounds that increase the levels of L-Phe such as precursors of L-Phenylalanine, which serves to inhibit the feedback inhibition of GTPCH1 by GFRP and BH4.

Yet another embodiment of the invention relates to agents or compounds that modulate the activity or the synthesis of GFRP. An embodiment of the invention includes agents or compounds that inhibit the activity of GFRP. Another embodiment of the invention includes the use of siRNA, small molecules, antibodies, antibody fragments and the like to inhibit the synthesis of GFRP.

#### VI. Biopterin Assays

The concentration of total biopterin and oxidized biopterin in plasma, blood and other tissues are determined based on the method of Fukushima et al (Anal. Biochem. 102:176 (1980)). Biopterin has four different forms including two forms of reduced biopterin, R-tetrahydrobiopterin (BH4) and quinonoid R-dihydrobiopterin (q-BH2) and two forms of oxidized biopterin, dihydrobiopterin (BH2) and biopterin (B). Of these four forms, only the reduced forms of biopterin have coenzymatic activity. Reduced biopterin is converted to B by iodination under acidic conditions, whereas under alkaline conditions, it is converted to pterin. Oxidized biopterin is converted to B by iodination under acidic and alkaline conditions. By taking advantage of this property, the amount of total biopterin is determined upon iodination under acidic conditions and that of oxidized biopterin is determined upon iodination under alkaline conditions, so that the amount of reduced biopterin is calculated from the difference in quantity thereof. When used as a coenzyme, BH4 is converted to q-BH2. The q-BH2 is immediately converted to BH4 by dihydropterine reductase or if not reduced, it is oxidized to BH2 or DHPT. Because it is difficult for biopterin to exist in the form of q-BH2 in vivo, the reduced biopterin may well be displaced as BH4.

Plasma and whole blood samples collected are immediately subjected to oxidation with acidic oxidizing solution (0.6N HCl solution in water containing 0.6% potassium iodide (KI), 0.3% iodine (I2) and 0.6N trichloroacetic acid (TCA)) and alkaline oxidizing solution (0.7N sodium hydroxide (NaOH)). Determination of B is performed by HPLC and radioactivity is measured using a liquid scintillation counter.

Measurement of BH4 using Reverse Phase HPLC (RP) Coupled with Tandem Mass Spectrometry (LC/MS/MS): The combined use of reverse phase high performance liquid chromatography (RP) and tandem mass spectrometry (LC/MS/MS) was shown to be selective for BH4 in human plasma, sensitive for BH4 in the range of 5-1000 ng/mL. The method is associated with about 50% conversion of BH4 due to oxidation during collection and storage. Samples are stable for greater than 3 months in dipotassium salt of ethylenediaminetetraacetic acid (K<sub>2</sub>EDTA) plasma. Recovery from the pretreatment steps is about 75%. The accuracy and precision of the method was determined to have coefficient of variation (CV) % below 15% (20% at the lower limit of quantitation, LLOQ).

The combined use of HPLC and tandem mass spectrometry was shown to be an improvement over HPLC alone in determining the BH4 test article because of: (1) its increased selectivity for drug-BH4 (whereas HPLC measures total biopterin), (2) broader qualitative range, (3) established con-

version ration, (4) extensive characterization and proven utility in human subjects, and (5) novel and useful measurement in different species and matrices.

The improved method comprises the following steps. Samples of blood, plasma, tissue homogenates, or urine are subjected to acidic or alkaline oxidation. With acidic oxidation, (1) the samples are treated with potassium chloride (KCl), hydrochloric acid (HCl) or TCA for an hour; (2) the acid oxidized samples are then subjected to iodometry; (3) the samples are run through an ion exchange column; (4) total biopterin comprising BH4, q-BH2 (which is immediately reduced in vivo to BH4 such that the measured reduced biopterin is based mainly upon BH4), BH2, and B are measured using HPLC and tandem mass spectrometry. With alkaline oxidation, (1) the samples are treated with KI, I2 or NaOH for an hour; (2) the alkaline oxidized samples are then subjected to acidification with HCl or TCA; (3) subjected to iodometry; (4) the samples are run through an ion exchange column; (5) oxidized biopterin comprising BH2 and B are measured; (6) different species are measured using HPLC and tandem mass spectrometry; and (7) the amount of reduced biopterin (BH4+q-BH2) is calculated as the difference between total biopterins less the oxidized form.

Flow charts for biopterin measurement and assay validation summary are provided in FIGS. 16 and 17.

#### Optimized Assay

An HPLC method using Electrochemical Detection (ECD) and Fluorescence (FL) detection is advantageous as it allows for the measurement of each of the discrete biopterin compounds (BH4, BH2 and B) as well as analogs.

BH4 is a cofactor for the enzyme system nitric oxide synthase (NOS), which produces nitric oxide (NO). The production of NO is important for maintaining vascular homeostasis. When intracellular levels of BH4 are limited, NO production is diminished (due to decreased NOS activity) and leads to the generation of the damaging free radical superoxide ( $O_2^-$ ). Excess  $O_2^-$  can lead to endothelial dysfunction and may contribute to the oxidation of BH4 to BH2. A low ratio of BH4 to BH2 may promote endothelial injury, whereas a high BH4 to BH2 ratio may promote endothelial health. Therefore, characterizing the BH4 to BH2 ratio may serve as a predictor of endothelial health.

The concentrations of different biopterins (BH4, BH2 and B) or analogs are determined by initially using reverse phase HPLC for separation, followed by ECD and FL detection.

BH4, which is a redox-sensitive, non-fluorescent molecule, is measured using ECD. BH4 (and analogs thereof) are measured using ECD in which BH4 (or analog) is oxidized by electrode 1 to a quinonoid dihydrobiopterin form (e.g., qBH2), a short-lived dihydrobiopterin intermediate, which is then reduced back to BH4 (or analog) at electrode 2. The detector then uses the current generated by this reduction reaction to determine the concentration of BH4 or analog thereof (endogenous qBH2 is negligible).

BH2, B, and analogs thereof can be measured in the same injection by fluorescence detection. Post-ECD oxidation of BH2 or an analog thereof using a conditioning guard cell at the optimum potential oxidizes BH2 or an analog thereof to B or the corresponding biopterin analog. This is desirable because BH2 is not fluorescently active or easily measured and must be converted to B, which is easily measured using fluorescence. Endogenous BH2, once converted to B, and endogenous B are distinguished from one another by two separate fluorescent peaks, due to the different retention times on the HPLC column for each molecule.

In total the methods can be used to measure the species BH4, BH2, and B, and analogs thereof. The biopterins pref-

erably are measured using a 2% MeOH-containing mobile phase, as described herein. Biopterin analogs, such as valine biopterin derivatives, may be better suited to higher methanol contents in the mobile phase, e.g. a 10% MeOH-containing mobile phase.

Thus, a method for detecting biopterins in a mixture of biopterin species can include (a) separating biopterin species in the mixture by reverse phase HPLC; and in the case of BH4 and analogs thereof, (b1) performing electrochemical detection by oxidizing the BH4 and analogs thereof present by a first electrode to quinonoid dihydrobiopterin forms, followed by reducing the quinonoid forms back to BH4 and analogs thereof present at a second electrode, and measuring current generated by the reduction reaction to determine the concentration of species; and/or (b2) in the case of BH2, analogs thereof, biopterin, or analogs thereof, measuring such species by fluorescence detection following post-column oxidation of BH2 species to biopterin. Preferably, the mobile phase is one disclosed herein.

In one embodiment, the preferred mobile phase includes sodium acetate, citric acid, EDTA, and 1,4-dithioerythritol (DTE) with methanol. Preferred concentrations are 50 mM sodium acetate, 5 mM citric acid, 48  $\mu$ M EDTA, and 160  $\mu$ M DTE with 2% methanol.

## VII. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Concentration Time Curve for Biopterin in Plasma After a Single Oral Dose in the Rat

The purpose of this study was to assess the pharmacokinetics of BH4 after a single oral administration in rats. Single doses of BH4 (10 and 100 mg/kg) were administered orally to male Sprague Dawley rats (6 weeks old) under fasting conditions.

The maximum total biopterin concentrations in plasma 2 hrs and 1 hr post-dosing were 108 ng/ml (i.e., about 3 $\times$  the endogenous level) and 1227 ng/ml (i.e., about 30 $\times$  the endogenous level), respectively (FIG. 18). Thereafter, biopterin had an elimination half-life ( $t_{1/2}$ ) of about 1.1 hr, returning to the endogenous level 9 hrs post-dosing for the 10 mg/kg dose and 24 hrs post-dosing for the 100 mg/kg dose (FIG. 18).

The bioavailability (F) after a 10 and 100 mg/kg oral administration were 6.8% and 11.8%, respectively, based on the area under the plasma concentration-time curve ( $\Delta$ AUC) obtained by subtracting the endogenous level during a 10 mg/kg intravenous administration. Rate of GI absorption were 8.8% when measured using radioactive markers in urine. An estimate of the actual value would be approximately 10% oral bioavailability based on these data.

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The ratio of reduced biopterin to total biopterins in plasma (i.e., the reduced-form ratio) was relatively static (73%-96%) (FIG. 19).

## Example 2

Concentration Time Curve For Biopterin In Plasma  
After Single Oral Dose to Monkey

The purpose of this study was to assess pharmacokinetics of sapropterin after a single oral administration in cynomolgus monkeys. A single dose of sapropterin (10 mg/kg) was administered orally to female cynomolgus monkeys (3/group) under fasting conditions.

## Results

The total plasma biopterin concentration ( $\Delta C$ ) reached its maximum value 3 hrs post-dosing (344 ng/ml, approximately 20 $\times$  endogenous levels) (FIG. 20). The plasma elimination half-life of biopterin was approximately 1.4 hrs, returning to the endogenous level within 24 hrs post-dosing. The ratio of reduced biopterin to total biopterins was nearly constant during the test period. The bioavailability (F) following a 10 mg/kg oral administration to female monkeys was about 9%, measured as  $\Delta AUC$  oral/iv ratios (FIG. 21).

## Example 3

Relative Bioavailability of Tetrahydrobiopterin  
(BH4) Administered After Dissolution of Tablet(s) in  
Water or Administered as Intact Tablet(s), and Effect  
of Food on Absorption in Healthy Subjects

## Objectives

The primary objectives of the study were: (1) to evaluate the relative bioavailability of tetrahydrobiopterin (BH4, sapropterin dihydrochloride) when administered after dissolution of tablet(s) in water or administered as intact tablet(s); (2) to compare the effect of food on the bioavailability of BH4 in healthy subjects. The secondary objective of the study was to assess the safety and tolerability of single oral doses of BH4 in healthy subjects.

## Methodology

This study was an open-label, randomized, three-treatment, six-sequence, three-period crossover study in which 30 subjects were to complete 3 single-dose dosing periods and were randomized to one of six sequence groups (Groups 1, 2, 3, 4, 5, and 6):

- Group 1: a, b, c
- Group 2: b, c, a
- Group 3: c, a, b
- Group 4: a, c, b
- Group 5: b, a, c
- Group 6: c, b, a

where all dosing groups received BH4 10 mg/kg orally as follows:

- a: administered after dissolution of tablet(s) in water given in fasting under fasting conditions
- b: administered as intact tablet(s) given in fasting under fasting conditions
- c: administered as intact tablet(s) given 30 minutes after beginning to ingest a high-calorie, high-fat meal in fed conditions

Each subject received a single dose of 10 mg/kg of BH4 during each treatment period. A washout period of at least seven days separated each dose administration. A post-study assessment was performed 5-7 days after discharge of the third treatment period. Blood samples for Pharmacokinetic

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(PK) analysis were drawn at scheduled collection times during each study period: within 30 minutes prior to dose, and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post-dose.

## 5 Dose and Mode of Administration

BH4 tablets were administered as 10 mg/kg dosages per treatment period. Tablets were administered by a) dissolution in water given in a fasted state, b) as intact tablets given in a fasted state, or c) as intact tablets given in a fed state.

10 Each dose of study drug was prepared and administered in liquid (solution) form mixed with water. The water supplied was ambient temperature tap water. Dosing solutions were prepared within 15 minutes of scheduled dose time. Dissolution of the tablet in liquid took approximately 1 to 3 minutes.

15 The tablets were broken up or crushed in the dosing cup prior to dissolution in order to improve dissolution rate.

At the designated morning dosing time, BH4 was administered orally as the number of tablets equivalent to a 10 mg/kg dose, dissolved in 120 mL of water or orange juice. Each subject was observed closely as the entire 120 mL dose was consumed within 15 minutes of preparation. Immediately after the dose had been consumed, the dosing cup was rinsed with 60 mL of water and the subject consumed the rinse. A second 60 mL water rinse was added to the dosing cup and then the subject consumed the second rinse. The entire dosing procedure was completed in a 1-minute time period. A qualified staff person inspected the dosing cup and each subject's mouth immediately after completion of the dose to ensure that the entire dose was consumed. Alternatively, the subject swallowed a pill containing the BH4 rather than dissolving it in water. For each individual, the dosing periods occurred with a minimum of 7 days between doses.

## Food Intake Schedule

A snack was served the evening of check-in. All subjects were then required to fast for at least 10 hours prior to dosing. Fasting Conditions

Subjects receiving treatments administered under fasting conditions were dosed after they completed a minimum 10-hour overnight fast.

20 The subjects continued to fast for 4 hours post dose. Water was allowed ad lib during the study except for 1 hour prior through 1 hour post-dose. Standardized meals were provided at approximately 4 and 10 hours after drug administration and at appropriate times thereafter.

## 45 Non-Fasting Conditions

Subjects receiving treatments administered under non-fasting conditions were dosed after consuming a high-calorie, high-fat breakfast meal. Subjects received the following standard high-fat (approximately 50% of total caloric content of the meal), high-calorie (approximately 1000 calories) breakfast that began 30 minutes prior to scheduled administration of the dose and ended (last bite taken) within 5 minutes prior to dosing.

- 2 eggs fried in butter
- 2 strips of bacon
- 2 slices of toast with butter
- 4 ounces of hash brown potatoes
- 8 ounces of whole milk

This meal contained approximately 150 protein calories, 250 carbohydrate calories, and 500-600 fat calories. An equivalent meal was substituted with documentation of the menu and caloric contents.

The subjects then fasted for 4 hours post dose. Water was allowed ad lib during the study except for 1 hour prior through 1 hour post-dose. Standard meals were provided at approximately 4 and 10 hours after drug administration and at appropriate times thereafter.



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## Duration of Treatment

Three single-dose treatment periods were each separated by a minimum of 7 days.

A follow-up visit was conducted 5 to 7 days after the last treatment visit.

## Safety Variables: Evaluation and Methods

Safety was evaluated for all subjects who take at least one dose of BH4.

## Efficacy and Safety Measurements Assessed and Flow Chart

Safety was evaluated by recording the incidence of adverse events, changes in 12-lead ECG parameters, vital signs and physical examination results, and changes in baseline in laboratory test values. The schedule for these assessments is shown in FIG. 22.

## Physical Examinations and Vital Signs

Each subject underwent a routine physical examination by the study investigator. The physical examination included evaluation of head, eyes, ears, nose, throat, neck, heart, chest, lungs, abdomen, extremities, peripheral pulses, neurologic status, skin, and other physical conditions of note are evaluated. This study protocol did not require genitourinary examinations.

Height (in centimeters) and weight (in kilograms) were measured and body mass index (BMI) was calculated ( $BMI = \text{weight (kg)} / [\text{height (m)}]^2$ ).

Blood pressure was measured in the sitting position according to the American Heart Association recommendations. Subjects were at rest with their feet on the floor for 5 minutes in the sitting position when blood pressure was measured.

Heart (pulse) rate was measured while the subject was in the sitting position.

A standardized 12-lead electrocardiogram (ECG) recording was taken at screening and at study discharge. ECGs were evaluated by a qualified investigator. Copies of the ECG and evaluation reports were kept as part of each subject's file.

The medical history, clinical laboratory test results and ECG tracing(s) were reviewed and evaluated by the Principal Investigator to determine clinical eligibility of each subject to participate in the study.

## Clinical Laboratory Assessments

## Hematology:

The following were evaluated: hemoglobin, hematocrit, total and differential leukocyte count, red blood cell (RBC) and platelet count.

In addition, blood was tested for Hepatitis B Surface Antigen, Hepatitis C Antibody and Human Immunodeficiency Virus (HIV).

## Chemistry:

The following were evaluated: albumin, blood urea nitrogen (BUN), creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ), lactic dehydrogenase (LDH), uric acid, and glucose.

## Urinalysis:

The following were evaluated by the urine dipstick method: pH, specific gravity, protein, glucose, ketones, bilirubin, blood, nitrite, and urobilinogen. If protein, occult blood, or nitrite values are out of range, a microscopic examination is performed.

Urine samples were also tested for drugs of abuse (amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine and opiates).

## Adverse Events

In this study, an adverse event (AE) was defined as any untoward medical occurrence in a subject or clinical investigation subject administered BH4, at any dose, whether or not

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it has a causal relationship with the event. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of BH4, whether or not related to BH4. This definition included intercurrent illnesses or injuries and exacerbation (increase in frequency, severity or specificity) of pre-existing conditions.

The reporting period for AEs began with the first administration of BH4. The reporting period for serious adverse events (SAEs) began earlier, from the time of the signing of the Informed Consent. SAEs were defined later in this section. The investigator monitored all AEs until resolution or, if the AE was determined to be chronic, a cause was identified. If an AE remained unresolved at the conclusion of the study, the PI and Medical Monitor made a clinical assessment as to whether continued follow-up of the AE was warranted, and documented the results. Assessment of severity was one of the responsibilities of the investigator in the evaluation of AEs and SAEs. The investigator was responsible for applying his or her clinical judgment to assess the causal relationship of each AE to BH4.

## Serious Adverse Events

A serious adverse event (SAE) was defined as any AE that has at least one of the following outcomes:

Resulted in death

Was life-threatening, that is, placed the subjects at immediate risk of death from the event as it occurred

This definition did not include a reaction that, had it occurred in a more severe form, might cause death

Required inpatient hospitalization or prolongation of existing hospitalization

Admission of a subject to the hospital as an inpatient as a result of an AE, even if the subject was released on the same day, qualified as hospitalization. An emergency room visit did not constitute hospitalization.

Resulted in persistent or significant disability or incapacity

An event qualified as resulting in a persistent or significant disability or incapacity if it involved a substantial disruption of the subject's ability to carry out usual life functions. This definition was not intended to include experiences of relatively minor or temporary medical significance.

Was a congenital anomaly or birth defect, that is, an AE that occurred in the child or fetus of subject exposed to study drug prior to conception or during pregnancy

Was an important medical event that did not meet any of the above criteria, but could jeopardize the subject or required medical or surgical intervention to prevent one of the outcomes listed above.

More than one of the above outcomes could apply to any specific event.

## Appropriateness of Measurements

The measures of safety in this study were routine physical examinations, vital signs, adverse event incidence and severity, and clinical and laboratory procedures.

## Drug Concentration Measurements

Blood (plasma) pharmacokinetic (PK) characteristics were assessed after each dose of study medication. All subjects remained seated in an upright position for 4 hours post-dose. The blood samples were drawn within 30 minutes prior to dose and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, and 24.0 hours post dose. Samples were collected in appropriately labeled 6 mL  $\text{K}_2$ -EDTA purple top Vacutainer® tubes. Blood samples were centrifuged at approximately 3000 rpm at 4° C. for 10 minutes. From the resulting plasma, exactly 1 mL was removed from each sample using a pipet, and placed into an aliquot tube containing 0.1% w/v dithioerythritol. The sample was capped and

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vortexed for approximately 10 seconds using a VWR Mini Vortexer at speed 6. After completion of these steps, the sample was flash frozen in an isopropyl/dry ice bath and placed in a  $-70^{\circ}\text{C}$ . freezer pending analysis.

Approximately 80 mL of blood was drawn during each treatment period (5 mL per timepoint) for the PK analysis. Pharmacokinetics:

Pharmacokinetic (PK) analysis of plasma BH4 concentration-time data was performed using non-compartmental methods to obtain estimates of the following PK parameters:

Peak plasma concentration ( $C_{max}$ ) and time to peak concentration ( $T_{max}$ ), obtained directly from the data without interpolation;

$\lambda_z$ , the apparent terminal elimination rate constant, determined by log-linear regression of the terminal plasma concentrations;

Area under the plasma concentration-time curve from time zero to the time of last measurable concentration [AUC(0-t)], calculated by the linear trapezoidal method;

The apparent elimination half-life ( $t_{1/2}$ ), calculated as  $0.693/\lambda_z$ ;

Area under the plasma concentration-time curve from time 0 to infinity [AUC(inf)] where  $\text{AUC(inf)} = \text{AUC(0-t)} + C_t/\lambda_z$  and  $C_t$  is the last measurable concentration.

#### Estimation of Absorption Rate

Subjects were given a 10 mg/kg oral or intravenous dose of BH4, followed by serial measurements of plasma total bioprotein concentration to determine the rate of BH4 absorption from the gastrointestinal tract from the area under the plasma total bioprotein concentration increase ( $\Delta\text{Cp}$ )-time curve ( $\Delta\text{AUC}$ ). It was anticipated that a lower dose of BH4 was required when administered intravenously in comparison with BH4 administered orally to achieve the same level of bioavailability. For example, it may require 10 mg/kg of BH4 given orally to achieve the same level of bioavailability as 1 mg/kg BH4 administered intravenously. Because the manner of administration enhanced bioavailability, it may require only 5 mg/kg of BH4 to achieve the same level of bioavailability as a 1 mg/kg IV dose of BH4.

The rate of BH4 absorption from the gastrointestinal tract was estimated from the area under the plasma total bioprotein concentration increase ( $\Delta\text{Cp}$ )-time curve ( $\Delta\text{AUC}$ ) after the administration BH4 using the following formulas:

Estimation from AUC

Absorption rate(%)=

$$\frac{(\Delta\text{AUC after p.o. dose}/\Delta\text{AUC after i.v. dose}) \times (\text{i.v. dose}/\text{p.o. dose} \times 100)}{1}$$

#### Statistical Methods:

Comparison of the pharmacokinetic parameters  $C_{max}$ , AUC(0-t), and AUC(inf) for BH4 was conducted using an analysis of variance (ANOVA) model with sequence, subject within sequence, treatment, and period as the classification variables using the natural logarithms of the parameters as the dependent variables. The comparisons of interest were between the dissolved and intact tablet in the fasted state and the intact tablet in the fed and fasted states.

The data from all subjects completing at least two study periods were included in the PK statistical analyses. All subjects receiving at least one dose of study drug were included in the safety analyses.

All PK and associated statistical analyses were done using SAS® for Windows® Version 9.1.3 or higher.

To provide sufficient power to meet the objectives of the study, a sample size of approximately 30 subjects, each with 3 treatment periods, was considered adequate to provide esti-

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mates of the differences comparisons of interest. No formal sample size calculation was conducted.

#### Results

##### Pharmacokinetics

##### Intact Versus Dissolved Tablets

Mean plasma concentrations of BH4 were lower when BH4 was administered as a dissolved tablet compared to the intact tablet (FIGS. 23 and 24). Mean  $C_{max}$  higher for the intact tablet as were mean values for AUC(0-t) and AUC(inf) (FIG. 25). The geometric mean ratios, intact-to-dissolved tablet, ranged from 118% to 121% and the upper limits of the associated 90% confidence intervals were greater than 125% (FIG. 26), indicating a statistically significant increase in absorption when the intact tablet is administered with a high-calorie, high-fat meal difference in absorption between dissolved and intact tablet administration. The median and range for  $T_{max}$  were essentially the same for the dissolved and intact tablets (FIG. 25), suggesting that the increase seen with the intact tablet was in the extent but not the rate of absorption.

##### Effect of High-Calorie, High-Fat Food on Drug Absorption

As expected, administration of the intact tablet with a standard high-fat high-calorie meal resulted in a substantial increase in the mean plasma BH4 concentrations (FIG. 23) and mean values for  $C_{max}$ , AUC(0-t), and AUC(inf) (FIG. 25). The geometric mean ratios (fed-to-fasted) ranged from 126% to 139% (FIG. 26) and, consequently, the upper limits of the associated 90% confidence intervals were greater than 125%, indicating a statistically significant difference in the effect of food on absorption compared to intact tablets. The median and range for  $T_{max}$  were essentially the same under fed and fasted conditions (FIG. 25), suggesting that the increase seen with food was in the extent but not the rate of absorption of absorption.

##### Safety:

There were no serious adverse events (SAEs) in this study. Five (5) subjects reported a total of 9 adverse events (AEs). Eight (8) of these 9 AEs were assessed as mild and 1 was assessed as moderate in severity. The most common AE was headache; 1 subject experienced a moderate headache which was assessed as unrelated to the study drug, and one subject experienced mild headache on two occasions, both of which were assessed as possibly related. In all, five events were judged to be unrelated and 4 were judged to be possibly related to the study drug. Study exit assessments, ECG and physical examination evaluations were completed with no clinically significant findings.

#### CONCLUSIONS

Administration of BH4 as an intact tablet resulted in an approximate 20% increase in the extent of absorption compared to a dissolved tablet.

Administration of BH4 as an intact tablet with a high-calorie, high-fat meal under fed conditions resulted in an approximate 30% increase in the extent of absorption compared to fasted conditions.

No clinically significant issues and safety parameters safety issues were identified in this study population. There were no AEs considered serious in this study. Among the 9 AEs reported, all but one, an instance of headache, was mild, and it was assessed to be unrelated to the study drug. Instances of fatigue and headache were the only AEs which were possibly related to the study drug, but and these were assessed as mild in severity.

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Example 4Formulation Approaches to Enhance Bioavailability  
of BH4

Two control formulations (BH4 intravenous formulation and BH4 tablet for oral solution) and six test formulations were selected for testing in animal studies. Each formulation prototype contained 80 mg or 100 mg of BH4.

## BH4 Intravenous Formulations

Table 3 lists the composition of an intravenous formulation. BH4 was passed through a #20 mesh stainless screen before use while mannitol was used as received. This formulation was filled as a powder in a bottle and constituted with sterile water for injection prior to administration. Each bottle contained 100 mg of BH4 and 5 g of mannitol in a clear polyethylene terephthalate copolyester (PETG) bottle with a white high-density polyethylene (HDPE) screw top closure. Prior to administration, the formulation was constituted with 100 mL of sterile water for injection to yield a final concentration of 1 mg/mL. The IV formulation was supplied as a dry powder in a bottle, and each bottle contained the API and mannitol. The powder was dissolved in sterile water for injection and filtered prior to administration by IV route.

TABLE 3

Composition of BH4 IV Formulation		
Ingredients	% (w/v)	mg/mL
BH4	0.1	1.0
Mannitol (low in endotoxin), USP/Ph. Eur.	5.0	50.0
Sterile water for injection	qs 100 mL	qs 1 mL

## BH4 Tablet for Oral Solution

Table 4 lists the composition of an oral solution formulation. Ten (10) BH4 tablets (100 mg) were placed into a 125 mL graduated PETG bottle with a white HDPE closure. Prior to administration, the formulation was constituted with 100 mL of sterile water for injection to yield a final concentration of 10 mg/mL.

TABLE 4

Composition of BH4 Tablet, 100 mg		
Ingredients	% (w/w)	mg/tablet
BH4	33.33	99.99
Ascorbic Acid, USP/EP	1.67	5.01
Crospovidone, USP/EP	4.5	13.5
Dicalcium Phosphate Anhydrous, USP/EP	2.18	6.54
Mannitol (Parteck M 200), UPS/EP	57.06	171.18
Riboflavin universal, USP/EP	0.01	0.03
Sodium Stearyl Fumarate (PRUV), NF/EP	1.25	3.75
Total	100.00	300.00

## Formulation Prototype to Slow Gastro-Intestinal Motility

Table 5 lists the composition of a delayed gastric emptying time prototype. BH4 was passed through a #20 mesh stainless steel screen before use. The Capmul GMO-50 was melted in a 37° C. water bath. BH4 and ascorbic acid were weighed and added slowly to the melted Capmul while stirring vigorously. The solid dispersion was added dropwise into a size #2 capsule using a pipette. Three filled capsules were placed in a 100 cc high-density polyethylene (HDPE) bottle with a heat-induction seal closure.

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TABLE 5

Composition of BH4 Delayed Gastric Emptying Time Oral Capsule Formulation		
Ingredients	% (w/w)	mg/capsule
BH4	25	80
Glyceryl mono/di-oleate (Capmul GMO-50)	65	208
Ascorbic acid fine powder	10	32
Total	100	320

## Bioadhesive Prototype

Table 6 lists the composition of a bioadhesive prototype. All materials, except for Carbopol 71 G, were passed through a #20 mesh stainless steel screen. All materials were weighed and added to a plastic bag having a zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. The powder was compressed into a tablet using a 1/4" standard, round, concaved, plain-faced B tooling on a Globe Pharma MTCM-I manual press at 600 psi. Three tablets along with a silica gel desiccant canister were packaged in a 100 cc HDPE with a heat-induction seal closure.

TABLE 6

Composition of BH4 Bioadhesive Oral Tablet Formulation		
Ingredients	% (w/w)	mg/tablet
BH4	48.5	80.00
Carbopol 71 G	20.0	32.99
Polycarbophil (Noveon AA1)	20.0	32.99
Ascorbic acid fine powder	10.0	16.49
Sodium stearyl fumarate (PRUV)	1.5	2.47
Total	100.0	164.94

## Sustained Release Prototype

Table 7 lists the composition of a sustained release prototype tested in the monkey. All materials, except for Methocel K100M Premium CR, were passed through a #20 mesh stainless steel screen. All materials were weighed and added to a plastic bag having zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. The powder was compressed into a tablet using a 1/4" standard, round, concaved, plain-faced B tooling on a Globe Pharma MTCM-I manual press at 1200 psi. The tablets along with a Silica gel desiccant canister were packaged in a 100 HDPE bottle with heat-induction seal closure.

TABLE 7

Composition of BH4 Sustained Release Tablet Formulation		
Ingredients	% (w/w)	mg/tablet
BH4	53.5	80.00
Methocel K100M premium CR	35.0	52.34
Ascorbic acid fine powder	10.0	14.95
Sodium stearyl fumarate (PRUV)	1.5	2.24
Total	100.0	149.53

## Proton Donor Polymer Prototype

Table 8 lists the composition of a proton donor polymer prototype tested in the monkey. All materials, except for Eudragit L100-55 and Kollidon CL, were pre-screened using a #20 mesh stainless steel screen. All materials were weighed and added to a plastic bag having a zip-locking closure, which was then shaken for a few minutes until the mixture appeared uniform. A pre-weighed quantity of powder was filled into a size #2 capsule.

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A coating solution was prepared by dissolving Eudragit L100-55 and Carbowax PEG 4600 in Ethyl Alcohol. The Eudragit L100-55 and Carbowax PEG 4600 were weighed and added to a 125 mL graduated polyethylene terephthalate copolyester bottle (PETG). The Ethyl Alcohol was added to the PETG bottle, and it was placed in a 40° C. water bath with sonication until the solution was clear.

The powder-filled capsules were manually dipped into the coating solution and allowed to dry at 40° C. for 20 minutes. The dried capsules were weighed and then rolled in Syloid FP244 to remove residual tackiness. Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

TABLE 8

Composition of BH4 Proton Donor Capsule Formulation		
Ingredients Composition of Capsule	% (w/w)	mg/capsule
BH4	40.0	80
Eudragit L100-55	44.5	89
Crospovidone (Kollidon CL)	4.0	8
Ascorbic acid fine powder	10.0	20
Sodium stearyl fumarate (PRUV)	1.5	3
Total	100.0	200
Composition of Capsule Coating		
Ingredients Composition of Capsule Coating	% (w/w)	mg/capsule <sup>1</sup>
Eudragit L100-55	5.0	ND
Polyethylene glycol 4600 (Carbowax Sentry)	5.0	ND
Ethyl alcohol, 200 proof	100 mL	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains about 1 to 3% weight in polymer coating.  
ND = Not Determined

## Floating Delivery System

Table 9 lists the composition of a floating delivery system. All materials, except for Eudragit L100-55, were passed through a #20 mesh stainless steel screen. This tablet prototype comprised three layers; the middle layer contained the drug substance, which was sandwiched between two water-insoluble outer layers. The inner and outer materials were weighed and added separately to plastic bags having zip-locking closures, which were then shaken until the mixtures appeared uniform.

The two outer layers (12 mg each) and inner layer (14.5 mg) were weighed. One of the outer layers was added to the press, followed by the inner layer, and then the last outer layer. The layers were compressed into a tablet using a 3/16" round, beveled, plain-faced B Tooling on a Globe Pharma MTCM-I manual press at 200 psi.

A coating solution was prepared by dissolving Ethocel and PEG 4600 in an ethyl alcohol and purified water mixture. The ingredients were added to a PETG bottle, which was mixed and placed in a 40° C. water bath with sonication until the solution appeared clear.

The tablets were manually dipped in the coating solution and allowed to dry for 20 minutes at 40° C. Each tablet was re-weighed after coating. Seven (7) tablets were placed into each of the size #2 elongated capsules. Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

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TABLE 9

Composition of BH4 Floating Dosage Formulation		
Ingredients Outer Layers 1 and 3	% (w/w)	mg/tablet
Eudragit L100-55	49.5	5.94
Stearic acid	49.5	5.94
Sodium stearyl fumarate (PRUV)	1.0	0.12
Total	100.0	12.00
Ingredients Middle Layer 2	% (w/w)	mg/tablet
BH4	79.0	11.46
Stearic acid	10.0	1.45
Ascorbic acid fine powder	10.0	1.45
Sodium stearyl fumarate	1.0	0.15
Total	100.0	14.51
Ingredients 7 tablets in a Capsule	% (w/w)	mg/capsule
BH4	29.8	80.19
Stearic acid	34.6	93.31
Ascorbic acid fine powder	3.8	10.15
Eudragit L100-55	30.8	83.16
Sodium stearyl fumarate (PRUV)	1.0	2.70
Total	100.0	269.51
Ingredients Tablet Coating Solution	% (w/w)	mg/capsule <sup>1</sup>
Ethocel Standard 10 FP	5.0	ND
Carbowax PEG 4600	5.0	ND
Ethanol 200 proof	95.0 mL	ND
Purified Water	5.0 mL	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains about 3 to 8% weight in polymer coating.  
ND = Not Determined

## Gas Generating Floating Delivery System

Table 10. lists the composition of a gas generating floating delivery system. This formulation was composed of a core tablet containing the drug substance surrounded by a gas-generating outer layer. All materials, except for sodium bicarbonate and Methocel K100M CR, were pre-screened using a #20 mesh stainless steel screen. The inner core and outer layer materials were weighed and added separately to plastic bags having zip-locking closures, which were closed and shaken until the mixture appeared uniform. The blended powder for the inner core (35 mg) was compressed into a tablet using a 1/8" round, beveled, plain faced B Tooling on a Globe Pharma MTCM-I manual press at 800 psi.

A coating solution was prepared by dissolving using Ethocel and PEG 4600 in ethyl alcohol. The inner core tablets were manually dipped in the coating solution and allowed to dry for 20 minutes at 40° C. The blended powder for the outer layer (40 mg) was weighed. One half was added to the press, followed by the inner core tablet, and then the second half of the outer layer. The tablet was compressed using a 3/16" round, beveled, plain-faced B Tooling on a Globe Pharma MTCM-I manual press at 800 psi. Four (4) tablets were placed into each size #2 capsule.



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TABLE 10

Composition of BH4 Gas Generating Floating Dosage Formulation		
Ingredients	% (w/w)	mg/tablet
Inner tablet Core		
BH4	58.3	20.39
Ascorbic acid fine powder	19.4	6.80
HPMC K100MCR	19.4	6.80
Sodium stearyl fumarate (PRUV)	2.9	1.02
Total	100	35.01
Outer Tablet Layer		
HPMC K100MCR	46.1	18.46
Citric acid anhydrous	34.2	13.68
Sodium bicarbonate	17.1	6.84
Sodium stearyl fumarate	2.6	1.03
Total	100	40.01
Four tablets in a Capsule		
BH4	27.2	81.55
Ascorbic acid fine powder	9.1	27.18
HPMC K100MCR	33.7	101.03
Citric acid anhydrous	18.2	54.70
Sodium bicarbonate	9.1	27.35
Sodium stearyl fumarate	2.7	8.18
Total	100	299.99
Coating Solution		
Ethocel Standard 10 FP	5.0	ND

<sup>1</sup>Following capsule coating and drying in the oven at 40° C., the capsule gains weight in polymer coating.  
ND = Not Determined

## Bioadhesive Granule Prototype

Table 11 lists the composition of a bioadhesive granule prototype. All materials, except for Methocel K100M CR, were pre-screened using a #20-mesh stainless steel screen. All materials, except for the sodium stearyl fumarate (PRUV), were weighed and placed into a size #1 granulator bowl (LB Bohle Mini Granulator BMG). The powder was mixed at an impeller speed of 300 rpm and a chopper speed of 2500 rpm for five minutes until the mixture appeared uniform. Maintaining the impeller and chopper speeds, 5 mL of ethyl alcohol was added dropwise to the mixture until granules formed. The wet mass was removed from the granulation bowl and screened through an 18-mesh stainless steel screen. The granules were collected and placed in a 40° C. oven to dry for one hour. The loss on drying of the granules was determined to be 1.93% after one hour of drying. The granules were weighed and placed into a plastic bag having a zip-locking closure. Sodium stearyl fumarate (PRUV) was added to the dried granules in the bag. The bag was closed and shaken until the sodium stearyl fumarate (PRUV) appeared evenly distributed among the granules. The granules were weighed (134 mg). Size 2 elongated capsules were filled with portions of the granules alternating with drops of partially hydrogenated vegetable oil (350  $\mu$ L). Three capsules were packaged in a 100 cc HDPE bottle with a heat-induction seal closure.

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TABLE 11

Composition of BH4 Bioadhesive Granule Capsule Formulation		
Ingredients	% (w/w)	mg/capsule
5		
BH4	60	80.00
Methocel K100M CR	19	25.33
Carbopol 971	10	13.33
Ascorbic Acid fine powder	10	13.33
Sodium Stearyl Fumarate (PRUV)	1	1.33
10 Pureco HSC-1 oil		350 $\mu$ L
Total	100	133.33

## In Vitro Drug Release

15 In vitro drug release testing from tablets was conducted according to the USP 27 apparatus II specifications using a Distek 2100C Dissolution Tester (Distek, Inc., North Brunswick, N.J.), along with an Agilent UV-Visible spectroscopy system (Agilent Technologies, Santa Clara, Calif.). The dissolution medium used for the release testing of BH4 was 900 mL of 0.1N HCl. During dissolution testing, the media in each vessel was maintained at 37° $\pm$ 0.5° C. and agitated at 50 rpm. A sample volume of 5 mL was taken at pre-determined time points. To determine the concentration of BH4 in the samples, 250  $\mu$ L of each sample was diluted with 500  $\mu$ L of 0.1N HCl and the absorption was measured at 265 nm using a UV spectrometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, Santa Clara, Calif.). The data were collected using ChemStation software (Rev. A.09.01[76], Agilent Technologies, Santa Clara, Calif.). All dissolution tests were performed in triplicate.

## Tablet Buoyancy Testing

35 The buoyancy of the floating prototype tablets was first determined by placing the tablets in plastic cups with 25-50 mL of 0.1N HCl. This test determined the time necessary for the tablets to float as well as the duration of their floating with no agitation. Those prototypes that floated for at least four hours were submitted for dissolution testing. During the dissolution testing, the buoyancy of the tablets was determined using the paddle method at a rotation speed of 50 rpm. The state of the tablets was checked visually at various time points.

## Disintegration Testing

45 Disintegration testing was conducted according to the USP-27 disintegration test specifications using a Distek 3100 Series Disintegration Tester (Distek Inc., North Brunswick, N.J.). The disintegration media used was 900 mL of 0.1N HCl or 900 mL of 0.2M Potassium Phosphate pH 5.8. During the disintegration testing the media in the vessels was maintained at 37° $\pm$ 0.5° C. The tablets and capsules were visually inspected for disintegration.

## Tablet Hardness Testing

55 Tablet hardness was determined using a Dr. Schleuniger Pharmatron 8M Tablet Hardness Tester (Dr. Schleuniger® Pharmatron Inc., Manchester, N.H.). The tablets were placed into the jaw of the hardness tester, and the hardness was measured in kiloponds (Kp).

## Tablet Thickness

60 The thickness of the tablets was measured using a Mitutoyo Digimatic Indicator (Mitutoyo Absolute, Dr. Schleuniger Pharmatron Inc., Manchester, N.H.). The tablets were placed under the thickness gauge and the value indicated was recorded in millimeters (mm).

## Results and Discussion

65 Several prototypes were developed based on three concepts: gastroretentive, proton donor polymer to change intes-

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tinal pH, and sustained release dosage forms. The sections below described the formulation development of each prototype.

BH4 Intravenous Formulation—After sterile water constitution, the resulting solution was isotonic, pH 3.2 and contained 1 mg/mL of BH4, and was suitable for intravenous administration after sterile filtration through a 0.22 micron filter. Stability of the 1 mg/mL solution stored at ambient temperature was analyzed by HPLC every hour for three hours. The aged solution samples were then stored at  $-20^{\circ}\text{C}$ . and analyzed by HPLC after 2 weeks. FIG. 27 indicates that the solution was stable at ambient temperature for at least 3 hours after constitution and was stable for at least 2 weeks during storage at  $-20^{\circ}\text{C}$ .

#### BH4 Tablet for Oral Solution

Each bottle was packaged to contain ten (10) BH4 tablets, 100 mg. One-hundred (100) mL of purified water or sterile water for injection was added to the contents of each bottle. Following vigorous shaking of the bottle, the tablets rapidly disintegrated within 5 minutes. The resulting solution contained 10 mg/mL of BH4 for oral administration. Not all the ingredients in the tablet were soluble, and although the final solution appeared hazy or translucent, the active pharmaceutical ingredient was fully dissolved and the fine particulates were poorly soluble inactive ingredients.

#### Formulation Prototype to Slow Gastro-Intestinal Motility

This capsule formulation comprised of BH4 and ascorbic acid dispersed in a semi-solid fatty acid derivative (glyceryl mono/di-oleate, melting point of  $86^{\circ}\text{F}$ . ( $30^{\circ}\text{C}$ .)). Glyceryl mono/di-oleate (GMO) was also selected because GMO is chemically compatible with BH4. The dissolution profile depicted in FIG. 28 showed that over 90% of the drug was released in 2 hours and the dissolution profile remained unchanged after the capsules were stored at  $40^{\circ}\text{C}$ . for 57 days.

The drug dispersion in melted GMO, a semi-solid, was filled into hard gelatin capsules manually. The density of the semi-solid is greater than 1 g/mL, and it was possible to fill at least 80 mg dose at 25% drug loading in a size #2 capsule. It is expected that a size #0 capsule should be able to contain at least 200 mg of drug using the same formulation. Leakage of fatty acid from the capsule was observed during storage at  $40^{\circ}\text{C}$ . Preferably, capsules or softgel capsule formulations will be banded to avoid leaking of fatty acid during storage.

#### Bioadhesive Prototype

Many bioadhesives are made of either synthetic or natural polymers. Most of the current synthetic bioadhesive polymers are either polyacrylic acid or cellulose derivatives. Examples of polyacrylic acid-based polymers include but are not limited to carbopol, polycarbophil, polyacrylic acid (PAAc), etc. Cellulosics include but are not limited to hydroxypropyl cellulose and hydroxypropylmethyl cellulose (HPMC). Two bioadhesive prototypes were developed for testing in animal studies. The first prototype was a bioadhesive tablet formulation and the second a capsule containing bioadhesive granules.

Polycarbophil and carbomer polymers were selected for the development of the first bioadhesive tablet prototype. Carbopol 71 G is a granular form of carbomer and has good powder flow properties. All the batches of the fabricated tablets were of good quality with acceptable drug content (evident by close to 100% drug release in dissolution profiles) and acceptable hardness. Table 12 lists the representative tablet weight, thickness, and hardness of the bioadhesive prototype containing carbomer and polycarbophil.

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TABLE 12

Representative Tablet Weight, Thickness and Hardness for Bioadhesive Prototype containing Carbomer and Polycarbophil

Tablet Lot Number	Compression Pressure (psi)	Weight (mg)	Thickness (mm)	Hardness (Kp)
11210-83	600	165.4	5.24	10.5
11229-4	600	166.7	5.64	10.3
11229-4	800	164.1	5.27	14.4
11229-4	1000	164.9	5.12	18

HPMC and carbomer polymers were used for the development of the second bioadhesive granules. HPMC was selected because it is used as low-density hydrocolloid system and controlled drug release independent of pH. Granules were selected over tablet to increase the chance of bioadhesion by increasing the surface area of the dosage form. To facilitate the separation of the granules-filled capsule in dissolution medium, the granules were coated partially with hydrogenated oil. Without the oil coating, the granules hydrated and formed a capsule-shaped matrix without disintegrating into individual granules.

The release profiles of the two bioadhesive prototypes (tablet and granules) are shown in FIG. 29, which shows that the release profile of the tablet was longer than the granules. Drug release was about 90% in four hours and 95% in one hour for the tablet and granules bioadhesive dosage forms, respectively. Upon storage at  $40^{\circ}\text{C}$ . and ambient humidity for one month without moisture protection (no heat induction seal), the tablet prototype exhibited a slowdown in drug dissolution (FIG. 29). For prototypes containing carbomer, moisture protection precaution should be taken to protect the tablet from possibly hydrating prematurely. Sustained Release Prototype

Hydroxypropylmethylcellulose (HPMC) is used as a hydrophilic vehicle for the preparation of oral controlled drug delivery systems (Colombo, Adv. Drug Deliv. Rev. 1993, 11, 37). HPMC matrices are known to control the release of a variety of drugs (Chattaraj, et al. Drug Develop. Ind. Pharm., 1996, 22, 555; Pabon, et al., Drug Develop. Ind. Pharm., 1992, 18, 2163; Lee, et al., Drug Develop. Ind. Pharm., 1999, 25, 493; Basak, et al., Indian J. Pharm. Sci., 2004, 66, 827; Rajabi-Siabhooni, et al., J. Pharm. Pharmacol., 1992, 44, 1062). Various viscosity grades of HPMC (K4M, K15M and K100 M) to control the release of BH4 were evaluated in this study. The dissolution profiles of tablets made with various grades of HPMC are shown in FIG. 30. Drug release profiles were similar at 20% HPMC regardless of viscosity grade; over 80% of the drug was released in 2 hours. When HPMC polymer was exposed to aqueous medium, it underwent rapid hydration and chain relaxation to form gel layer (Naruhashi, et al., Pharm Res. 2003, 19:1415-1421). The HPMC at 20% may not form a substantial gel barrier layer to slow the release of BH4 significantly.

The dissolution profiles of tablets produced with varying concentrations (20% to 40%) of a high viscosity grade of HPMC (Methocel K100M CR) are presented in FIG. 30. A tablet containing 35% to 40% Methocel K100M CR was found to slow drug release for up to four hours whereas 20% HPMC released drug in two hours (FIG. 31). A tablet containing 35% HPMC (Methocel K100M) was selected as the prototype for testing in animal studies because it contained the least amount of HPMC required to slow the drug release for up to four hours. As such, the tablets were of good quality with acceptable drug content as evident by close to 100% drug release in dissolution profiles.



## Proton Donor Polymer Prototype

To increase the oral absorption of BH4, one approach is to stabilize the drug by decreasing the pH of the proximal small intestine. To manipulate intestinal luminal pH, Eudragit L100-55, a proton-releasing polymer commonly used for enteric coating, was selected. This polymer is not soluble under acidic conditions, and it becomes soluble and releases protons under weakly acidic (pH>5.5) to alkaline condition due to its carboxyl groups, thereby controlling the intestinal luminal pH to be acidic. Naruhashi, et al. (2003) found that pH in the lumen was decreased in a Eudragit L100-55 concentration-dependent manner and the absorption of cefadroxil and cefixime from the ileal loop was increased in the presence of the acidic polymer (Nozawa, et al., J. Pharm Sci. 2003, 92 (11), 2208-2216). Nozawa, et al (2003) showed that Eudragit decreased the pH in the intestinal loops, and increased the disappearance of both cefadroxil and cefixime from the loops.

Powder formulations containing BH4 and Eudragit L100-55 as shown in Table 8 were compressed into tablets and filled into capsules. The tablet formulation released about 27% drug in one hour in simulated gastric fluid (SGF) during dissolution testing. However, during disintegration testing, the tablet remained intact in SGF and pH 5.8 phosphate buffer (PB) for at least 2 hours. Even in the presence of a superdisintegrant (crospovidone or croscarmellose), the tablet failed to disintegrate. It is possible that the drug may be acidifying the Eudragit, creating a low micro pH environment such that the polymer remained unionized and insoluble.

The powder filled capsule drug-Eudragit formulation disintegrated rapidly in SGF. To target proton release in the proximal intestine, an enteric coat was applied to the capsule. Following capsule coating and drying in the oven at 40° C., the capsule gained about 1 to 3% weight in polymer coating. When tested using the USP dissolution apparatus II (paddle), dissolution medium 0.1 N HCl maintained at 37° C. at a rotational speed of 50 rpm, the coated capsule released about 25% of drug in one hour. Following 1 hour of acid (0.1 N HCl) pre-treatment, the coated capsule was placed in a USP disintegration Apparatus with 500 mL of pH 5.8 phosphate buffer maintained at 37° C., the coated capsule disintegrated in about 1 hour. The enteric-coated capsule prototype was selected over the tablet or the uncoated capsule because the enteric-coated capsule was more likely to deliver proton-releasing polymer to the target site.

## Floating Delivery System

Two floating delivery systems were developed. The first prototype was a floating multiple unit dosage form; the purpose of this dosage form was to increase the chance that one of the units will remain in the gastric region and hence prolong the gastric residence time of drugs. This dosage form consisted of seven triple layer tablets in a capsule; the middle layer contained the drug substance, which was sandwiched between two water-insoluble outer layers (FIG. 32). The outer layers contained stearic acid, a hydrophobic and water-insoluble fatty acid, which provided the necessary buoyancy to the floating tablet. Each tablet was manually coated with an alcoholic solution of ethylcellulose and polyethylene glycol MW 4600 (PEG). Ethylcellulose formed a water insoluble film around the tablet and PEG, which acted as a pore former, modulated the release rate. The dissolution profiles of tablets coated with ethylcellulose and various concentrations (20% to 40%) of PEG solutions are presented in FIG. 33. It was noted that the coated triple layer tablet achieved close to zero-order release kinetics. As expected, the drug dissolution rate increased as the concentration of PEG increased. The tablets floated in simulated gastric medium for at least four

hours during dissolution studies. Table 9 shows the composition of the formulation tested in animal studies.

The second prototype was a gas-generating dosage form. It was formulated in such a way that when it came in contact with acidic gastric contents, carbon dioxide was liberated and got entrapped in the swollen hydrocolloids, which provided buoyancy to the dosage form (FIG. 33). This formulation floated in simulated gastric medium for at least four hours during dissolution studies. However, for such a system to work consistently, the tablets have to be produced in a low humidity environment to prevent premature acid and base reaction. There could be potential interaction between BH4 and sodium bicarbonate in the tablet during storage. For these reasons, this dosage form was not tested in animal studies.

Six prototype test formulations that incorporated various formulation approaches including proton donor polymer to decrease intestinal pH, gastroretentive dosage forms, and sustained released formulations, were developed for animal bioavailability studies.

## Example 5

## Bioavailability of Novel BH4 Formulations

The objective of this study was to enhance the absorption of BH4 by developing dosage forms that increase the residence time of the drug in the gastrointestinal (GI) tract.

Methods: Three healthy cynomolgus monkeys weighing 3-4 kg were used in open, 8-period non-crossover study to determine the bioavailability of seven formulations compared to a control dissolved BH4 formulation. After an overnight fast, the monkeys received, on separate occasions, a single dose of 80 mg of the same novel formulation orally or intravenously with an interval of at least a 1 week washout period between the various novel formulations studied. For intravenous administration, blood samples were collected before dosing and then at 5, 15 and 30 min and 1.0, 2.0, 4.0, 6.0, 8.0, 12 and 24 hr post dose. For oral administration, blood samples were taken before dosing and then 15 and 30 min and 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12, and 24 hr following each dose. Following separation of the plasma by centrifugation, 200-μL aliquots of each sample were promptly transferred into individual tubes containing 0.1% DTE and frozen at -70° C. until ready for assay for total L-biopterin.

Study Formulations: The formulations administered are found in Table 13. Three of the formulations were conceptually designed to be gastroretentive via bioadhesive or floating mechanisms to increase GI residence time (carbomer-based, multi-particulate floating granules and bioadhesive granules). Other concepts were based on slowing GI motility to increase residence time of the formulation (glyceryl monooleate), reducing the pH of the small intestine and thereby enhancing BH4 chemical stability to enable absorption of intact drug (proton pump) or sustained delivery formulation to ascertain whether it will enhance absorption.

TABLE 13

Phase	Prototype	Dosage Form	Concept	Ingredients
Phase I	IV Formulation	IV solution, 1 mg/mL	Control	BH4, D(-)-Mannitol
Phase II	Kuvan Tablets for Solution	Oral Solution, 10 mg/ml	Control	BH4 tablets manufactured by Lyne (Lot# 140651)

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TABLE 13-continued

Phase	Prototype	Dosage Form	Concept	Ingredients
Phase III	Glycerol Mono Oleate	Capsule, 80 mg	Slow GI motility	BH4, Capmul GMO-50, Ascorbic Acid
Phase IV	Carbomer Prototype	Tablet, 80 mg	Gastro-retentive, Bioadhesive Sustained release	BH4, Carbopol 71G, Noveon AA1, Ascorbic Acid, PRUV BH4, Methocel K100M Premium CR, Ascorbic Acid, PRUV
Phase V	HPMC prototype	Tablet, 80 mg	Proton donor polymer to lower GI pH	BH4, Eudragit L100-55, Ascorbic Acid, Kollidon CL, PRUV, Coating (Eudragit L100-55, Carbowax PEG 4600, Ethyl Alcohol 200 proof)
Phase VI	Eudragit Prototype	Capsule, 80 mg	Gastro-retentive, floating	Inner Layer (BH4, Ascorbic Acid, Stearic Acid, PRUV), Outer Layer (Stearic Acid, Eudragit L100-55, PRUV), Coating (Ethocel Standard 10FP, Carbowax PEG 4600, 95% Ethanol)
Phase VII	Multi-floating units	Multiple tablets in capsule, 80 mg	Gastro-retentive, floating	Intergranular (BH4, Methocel K100M Premium CR, Carbopol 971, Ascorbic Acid), Extragranular (PRUV, Pureco HSC-1 oil)
Phase VIII	Bioadhesive Granules	Granules in capsule, 80 mg	Gastro-retentive, Bioadhesive	

Plasma Assay for Biopterin: BH4 concentrations in plasma were determined by using a validated, specific, reversed-phase LC/MS/MS method. The standard curve was linear over the concentration range of 50 ng/mL to 2500 ng/mL. The lower limit of quantitation for L-biopterin was 50 ng/mL with intraday precision shown by coefficients of variation less than 5%. L-biopterin is stable in frozen monkey plasma stabilized with 0.1% DTE at -70° C. until assayed. BH4 concentrations were calculated from the determined L-biopterin concentrations.

Pharmacokinetic and Statistical Analysis: Pharmacokinetic parameters were determined for plasma BH4 following the administration of the oral and intravenous formulations. The pharmacokinetic parameters are provided in Table 14.

TABLE 14

Phase, Formulation	AUC <sub>last</sub> (ng-hr/mL)	AUC <sub>∞</sub> (ng-hr/mL)	C <sub>max</sub> (ng/mL)	C <sub>last</sub> <sup>a</sup> (ng/mL)	T <sub>max</sub> (hr)	t <sub>1/2</sub> (hr)
2, dissolved tablet	641 (88)	805 (36)	93.6 (31.3)	9.60 (2.20)	2.33 (0.58)	11.7 (2.1)
3, glyceryl mono-oleate	716 (154)	858 (317)	133 (83)	6.47 (3.60)	2.00 (0)	12.1 (10.3)
4, bioadhesive polymer	593 (50.6)	648 (114)	108 (15)	4.46 (3.36)	2.67 (0.58)	6.89 (3.51)
5, sustained release	355 (134)	472 (36)	86.0 (43.1)	12.9 (12.4)	3.33 (0.58)	5.30 (1.73)
6, proton donor	276 (49.8)	282 (49)	68.3 (25.3)	2.97 (0.71)	3.33 (0.58)	1.59 (0.74)
7, floating dosage form	304 (78)	b	59.9 (31.8)	5.90 (0.94)	4.00 (2.00)	b
8, bioadhesive granulations	292 (79)	366 (40.6)	42.5 (12.6)	5.11 (2.43)	3.0 (0)	15.3 (8.2)

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## Results

The objective of this study was to identify formulations that enhance the bioavailability BH4 compared to the control dissolved tablet formulation. The mean plasma BH4 concentration-time profiles of the various dosage forms and the control formulation following the oral administration of BH4 are shown in FIG. 35, and the BH4 pharmacokinetic parameters derived from plasma drug concentration-time profiles are given in Table 14. The control formulation (phase 2) is the dissolved tablet.

As shown in FIG. 35, the glyceryl mono-oleate formulation provided the highest AUC<sub>last</sub> and AUC<sub>∞</sub> which are 716 ng-hr/mL and 858 ng-hr/mL respectively. The control dissolved BH4 tablet formulation exhibited AUC<sub>last</sub> and AUC<sub>∞</sub> which are 641 ng-hr/mL and 805 ng-hr/mL respectively (Table 14). The rank order of the formulations from the most to the least bioavailable is: glyceryl mono-oleate>dissolved tablet>bioadhesive polymer tablet>sustained release tablet>floating dosage forms>bioadhesive granulations capsule product>proton donor capsule product.

## Example 6

## Preparation of Intravenous Formulation of Tetrahydrobiopterin

## Preformulation Stability Evaluation

In general, the objective of this study was to evaluate the stability of BH4 in buffer solutions ranging in pH from pH 1 to 7 (See Table 15) and in the presence and absence of antioxidants and with or without inert gas in the reaction solutions (See Table 16).

TABLE 15

Components and Composition of Buffer Solutions to be used for BH4 Preformulation Stability Studies	
Components	Quantities
pH 1.2 Buffer (0.1N HCl)	
Concentrated HCl (12N)	8.33 mL
Sodium Chloride	2.92 g
Distilled/Deionized Water qs	1000 mL

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TABLE 15-continued

Components and Composition of Buffer Solutions to be used for BH4 Preformulation Stability Studies	
Components	Quantities
pH 2.1 Buffer (0.01N HCl)	
pH 1.2 (0.1N HCl) Buffer	100 mL
Sodium Chloride	7.79 g
Distilled/Deionized Water qs	1000 mL
pH 3 Buffer	
Phosphoric Acid, 15M, 85%	.347 mL
Sodium Monobasic Phosphate, anhydrous (NaH <sub>2</sub> PO <sub>4</sub> )	6.17 g
Sodium Chloride	6.16 g
pH 4 Buffer	
Acetic Acid, Glacial, 100%	2.38 mL
Sodium Acetate, Trihydrate	1.29 g
Sodium Chloride	8.22 g
Distilled/Deionized Water qs	1000 mL
pH 5 Buffer	
Acetic Acid, Glacial, 100%	.87 mL
Sodium Acetate, Trihydrate	4.78 g
Sodium Chloride	6.72 g
Distilled/Deionized Water qs	1000 mL
pH 6 Buffer	
4-Morpholineethanesulfonic (MES) Acid Monohydrate	4.99 g
MES Sodium Salt	5.75 g
Sodium Chloride	7.23 g
Distilled/Deionized Water qs	1000 mL
pH 7 Buffer	
Sodium Monobasic Phosphate, Monohydrate (NaH <sub>2</sub> PO <sub>4</sub> )	2.56 g
Sodium Dibasic Phosphate, anhydrous (Na <sub>2</sub> HPO <sub>4</sub> )	4.44 g
Sodium Chloride	2.18 g
Distilled/Deionized Water qs	1000 mL

TABLE 16

Composition of Buffer Solutions for Stability Studies Containing BH4 With or Without Antioxidant and whether Subjected to Gas Sparging or Not	
Study Group Number	
1 pH Study	2 Buffer + Ascorbic Acid Study
1 1 mg/mL BH4 in pH 1.2 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 1.2 Buffer
2 1 mg/mL BH4 in pH 2.1 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 2.1 Buffer
3 1 mg/mL BH4 in pH 3 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 3 Buffer
4 1 mg/mL BH4 in pH 4 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 4 Buffer

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TABLE 16-continued

Composition of Buffer Solutions for Stability Studies Containing BH4 With or Without Antioxidant and whether Subjected to Gas Sparging or Not	
Study Group Number	
3 Buffer + L- Cysteine Study	5 Buffer + Argon Sparging Study
1 1 mg/mL BH4 in pH 5 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 5 Buffer
2 1 mg/mL BH4 in pH 6 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 6 Buffer
3 1 mg/mL BH4 in pH 7 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 7 Buffer
4 1 mg/mL BH4 in pH 4 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 4 Buffer
5 1 mg/mL BH4 in pH 5 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 5 Buffer
6 1 mg/mL BH4 in pH 6 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 6 Buffer
7 1 mg/mL BH4 in pH 7 Buffer	1 mg/mL BH4 and 1 mg/mL Ascorbic Acid in pH 7 Buffer

More specifically, the influence of combining two antioxidants in the presence or absence of inert gas was evaluated at pH 4 to support the formulation of a liquid product, and at a pH 7 to ascertain the contribution of instability at physiologic pH to the low bioavailability of the compound in monkeys and humans (See Tables 17 and 18). The stability of BH4 is expected to be temperature-dependent. Therefore, the compound stability was evaluated at 2-8° C., 25° C., 30° C. and 37° C. to support the determination of predictive long-term shelf lives for the compound at different temperatures. Determination of the stability of the compound at the physiologic temperature of 37° C. provides data to support the estimation of the stability lifetime of a formulated oral dosage form in the absorptive regions of the GI tract.

TABLE 17

Composition of Buffer Solutions for the pH 4 Stability Study of BH4	
pH 4	pH 4
Buffer + Ascorbic Acid + L-Cysteine Study	Buffer + Ascorbic Acid + L-Cysteine + Argon Sparge Study
1 mg/mL BH4 and 1 mg/mL Ascorbic Acid and 1 mg/mL L-Cysteine in pH 4 Buffer	1 mg/mL BH4 + 1 mg/mL Ascorbic Acid + 1 mg/mL L-Cysteine in pH 4 Buffer and Argon-Sparged and Argon blanket-Sealed

TABLE 18

Composition of Buffer Solutions for the pH 7 Stability Study of BH4	
pH 7	pH 7
Buffer + Ascorbic Acid + L-Cysteine Study	Buffer + Ascorbic Acid + L-Cysteine + Argon Sparge Study
1 mg/mL BH4 and 1 mg/mL Ascorbic Acid and 1 mg/mL L-Cysteine in pH 7 Buffer	1 mg/mL BH4 + 1 mg/mL Ascorbic Acid + 1 mg/mL L-Cysteine in pH 7 Buffer and Argon- Sparged and Argon blanket-Sealed

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Proposed sampling times for studies to be conducted in various buffer solutions were estimated by comparing the half-life of a single study at pH 3.1 with data obtained by Davis, et al. (1988; Eur. J. Biochem. 173, 345-351, (1988)), in pH 6.8 Tris and phosphate buffers. The stability study of a pH 3.1 solution yielded an estimated  $t_{1/2}$  of 17769 min (12.3 days) and the work of Davis et al yielded a  $t_{1/2}$  of 10 min in phosphate pH 6.8 buffer and 14 min in pH 6.8 Tris buffer. These two studies suggest an order of magnitude reduction in half-life (i.e. an order of magnitude increase in reactivity) of BH4 for every one-fold increase in pH (see Table 19). Based on this approximation, pH 1.2 to pH 3 solutions were sampled weekly initially and sampling time corrections were made if necessary after the first 2 data points were collected. The estimated sampling times at 25° C. are provided in Table 19.

TABLE 19

Suggested Sampling Times at Various pH Based on Measured Half-life of BH4 and Theoretical Half-Lives Derived from Them			
pH	Measured $t_{1/2}$ (Min)	Estimated $t_{1/2}$ Based on $t_{1/2}$ Obtained at pH 3 (Min) <sup>a</sup>	Initially Suggested <sup>c</sup> Sampling Time
1.0	—	776900.0 (1234 days)	Every 7 days
2.0	—	177690.0 (123.4 days)	Every 7 days
3.0	17769.0 (12.34 days)	17769.0 (12.34 days)	Every 96 hours
4.0	—	1776.9 (1.23 days)	Every 12 hours
5.0	—	177.7 (0.12 days)	Every ½ Hour
6.0	—	17.7 (0.01 days)	Every 5 minutes <sup>d</sup>
6.8 <sup>b</sup>	10 (Phosphate) 14 (Tris)		
7.0	—	1.8	Every ½ minutes <sup>d</sup>

<sup>a</sup>Estimated  $t_{1/2}$  is based on changing by an order of magnitude, the half-life obtained at pH 3.0 for every one-fold change in pH. pH < 3 are increased upwards while pH > 3 are decreased downwards by an order of magnitude in a stepwise fashion to roughly match the pH 6.8 data obtained by Davis et al.

<sup>b</sup>Data obtained from Davis, et al. 1988; Eur. J. Biochem., 173, 345-351, (1988)

<sup>c</sup>Sampling can be modified

<sup>d</sup>Reaction solutions are sampled and quenched as fast as possible and require a stopwatch and 2 people, one sampling/quenching and the other accurately recording the time in a notebook in minutes and/or seconds

Studies were conducted in pH 1-7 buffer solutions and at 5° C., 25° C., 30° C. and 37° C. Although these studies were conducted in non-hermetically sealed containers, anti-oxidants alone (ascorbic acid or L-cysteine) or combined together (ascorbic acid+L-cysteine) reduced the rate of loss or degradation of BH4 (see FIG. 36 and FIG. 37). Sparging a solution containing both ascorbic acid and L-cysteine substantially enhanced the stability of BH4.

The rate of degradation of BH4 is concentration-dependent (see FIG. 38). Therefore high dose, highly concentrated formulations of BH4 were shown to require lower concentration of stabilizers for synergistic stabilization of the formulations.

This results demonstrate that formulation of long shelf-life, stable, liquid formulations can be produced according to the methods and compositions described herein, including sterile injectable liquids, oral liquids, and lyophilized and sterile powders for constitution formulations.

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## Example 7

## Liquid and Lyophilized Formulations of Tetrahydrobiopterin for Oral and Parenteral Use

## Example Compositions of Formulations

TABLE 20

Specific formulation buffered at pH 4 having ascorbic acid as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	10.00	1.00	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 21

Formulation buffered at pH 4.0 containing a combination of two stabilizers: ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	2.50	0.25	Antioxidant
Sodium Metabisulfite	2.50	0.25	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 22

Formulation buffered at pH 4.0 containing a combination of three stabilizers: L-cysteine, ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	1.00	0.10	Active substance
Ascorbic Acid	2.00	0.20	Antioxidant
Sodium Metabisulfite	2.00	0.20	Antioxidant
L-Cysteine	4.00	0.40	Antioxidant
Citric Acid	6.56	0.66	Buffering agent
Sodium Citrate, Dihydrate	5.53	0.55	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 23

Formulation buffered at pH 7.0 containing only ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	50.00	5.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.10	Buffering agent
Sodium Dibasic Phosphate	17.76	0.18	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

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Formulation buffered at pH 7.0 containing ascorbic acid sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.26	Buffering agent
Sodium Dibasic Phosphate	17.76	0.44	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 25

Formulation buffered at pH 7.0 containing ascorbic, sodium metabisulfite and L-Cysteine as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
L-Cysteine	10.00	1.00	Antioxidant
Sodium Monobasic Phosphate, Monohydrate	10.24	0.26	Buffering agent
Sodium Dibasic Phosphate	17.76	0.44	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

## High Dose Liquid Formulations

TABLE 26

Formulation buffered at pH 6.0 containing ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	0.10	Active substance
Ascorbic Acid	7.50	0.75	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 27

Formulation buffered at pH 6.0 containing a combination of two stabilizers: ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	5.00	Active substance
Ascorbic Acid	2.50	0.25	Antioxidant
Sodium Metabisulfite	2.50	0.25	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

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TABLE 28

Formulation buffered at pH 6.0 containing a combination of three stabilizers: L-cysteine, ascorbic acid and sodium metabisulfite			
Components	Amount (mg)	% Weight/Volume	Function
BH4	50.00	0.10	Active substance
Ascorbic Acid	2.00	0.20	Antioxidant
Sodium Metabisulfite	2.00	0.20	Antioxidant
L-Cysteine	1.00	0.10	Antioxidant
Citric Acid	5.30	0.53	Buffering agent
Sodium Citrate, Dihydrate	51.4	5.14	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 29

Oral formulation buffered at pH 3.0 citrate buffer and containing ascorbic acid only as stabilizer			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sucrose	200.00	20.00	Sweetener
Orange Flavor	1.00	0.10	Flavoring agent
Citric Acid	8.98	0.90	Buffering agent
Sodium Citrate, Dihydrate	2.13	0.21	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 30

Oral formulation buffered at pH 3.5 tartrate buffer and containing ascorbic acid and sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	5.00	0.50	Antioxidant
Sucrose	200.00	20.00	Sweetener
Grape Flavor	1.00	0.10	Flavoring agent
Tartaric Acid	1.34	0.13	Buffering agent
Sodium Tartrate Dibasic Dihydrate	8.39	0.84	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

TABLE 31

Oral formulation buffered at pH 3.5 in malic acid based buffer and containing ascorbic acid and sodium metabisulfite as stabilizers			
Components	Amount (mg)	% Weight/Volume	Function
BH4	10.00	1.00	Active substance
Ascorbic Acid	20.00	2.00	Antioxidant
Sodium Metabisulfite	15.00	1.50	Antioxidant
Sucrose	200.00	20.00	Sweetener
Apple Flavor	1.00	0.10	Flavoring agent
Malic Acid	3.07	0.31	Buffering agent
Sodium Malate Dibasic	4.91	0.49	Buffering agent
Water for Injection qs	1.00 mL	1.00 mL	Diluent

The foregoing formulated or compounded solutions are optionally sparged with an inert gas (e.g., argon or nitrogen) or carbon dioxide in the compounding tank and primary containers preferably are sealed in a blanket of inert gas or carbon dioxide to remove oxygen from the container headspace. The formulations can be scaled up to any volume by multiplying the component amounts by an appropriate scale up factor.



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## Example 8

LC/MS/MS Determination of Tetrahydrobiopterin  
(BH4) in Human Plasma by Measuring L-Biopterin  
Concentration upon Oxidation under Basic  
Conditions

Tetrahydrobiopterin (BH4) is a small molecule therapeutic for the treatment of patients with phenylketonuria (PKU). It is important to have an accurate and specific method to measure BH4 concentrations in human plasma. However, it is a challenge to quantify BH4 in human plasma because of its low endogenous concentration and instability. Under basic conditions, BH4 is oxidized into dihydrobiopterin (BH2) and ultimately L-Biopterin. Furthermore, the oxidation conversion ratio of BH4 to L-Biopterin is nearly constant up to 23 weeks. Therefore, by measuring L-Biopterin concentration upon oxidation under basic condition, and applying a molar conversion ratio, we can reliably determine the BH4 concentrations in human plasma.

Published methods are based on the classical method developed by Fukushima and Nixon (Anal. Biochem., 102, 176-188 (1980)) using HPLC with fluorescence detection. In the LC/MS/MS method, the human plasma sample was stabilized with antioxidant, spiked with an internal standard (IS) solution and basified with sodium hydroxide solution, then oxidized with iodine solution. Upon incubation in dark at room temperature, ascorbic acid is added to reduce the excess iodine. Oxidized samples were extracted by protein precipitation. L-Biopterin in the reconstituted extracts was analyzed by using reversed-phase HPLC with Turbo Ion Spray® MS/MS detection. Negative ions for L-Biopterin were monitored in MRM mode. Drug-to-IS peak area ratios for the standards were used to create a linear calibration curve using  $1/x^2$  weighted least-squares regression analysis.

The oxidation conversion ratio of BH4 to L-Biopterin was evaluated at multiple time-points: 0, 1, 2, 4, 8, 12 and 23 weeks, and found consistent in all the tested time-points with a nominal molar conversion ratio of 47.3% determined from the first three consecutive time-points. The difference between the conversion ratio at other time-points and the nominal value ranges from -2.3 to 6.3%. The LC/MS/MS method was validated to quantify L-Biopterin in K<sub>2</sub> EDTA human plasma in the linear calibration range of 5 to 1000 ng/mL (equivalent to 11 to 2114 ng/mL for BH4). The assay precision and accuracy was evaluated with quality control samples (QCs) and the results showed intraday precision between 4.7 to 14.5% CV; intraday accuracy between -7.1 to 7.4% nominal values; and interday precision and accuracy of 7.4 to 16.4% CV and -8.3 to 3.7% nominal values, respectively. The mean extraction recovery for L-Biopterin was 65.3%. In K<sub>2</sub> EDTA human plasma, L-Biopterin was found to be stable at room temperature for at least 4 hours and after 4 freeze thaw cycles, and at -70° C. for at least 275 days.

## Example 9

Determination of BH4/BH2/B Using HPLC with  
Electrochemical and Fluorescence Detection

A study was performed to develop a method of determining tetrahydrobiopterin (BH4), dihydrobiopterin (BH2) and biopterin (B) concentrations in human plasma using reverse phase high performance liquid chromatography (HPLC) with fluorescence detection (FD) and electrochemical detection (ECD). The method is based on Cai, et al. (Cardiovascular Research 55: 838-849, 2002).

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Stock solutions of BH4 (in 20 mM HCl), BH2 and B (in DMSO) were made to a final concentration of 10 mM and stored at -80° C. Calibration standard working solutions were prepared from stock solution at 100, 10, 7.5, 5, 2.5, and 1 nM in K2 EDTA human plasma modified by 0.1% (w/v) 1,4-Dithioerythritol (DTE). Quality control working solutions of BH4, BH2 and B were prepared at 5, 8, 25 and 50 nM in K2 EDTA human plasma modified by 0.1% (w/v) DTE and stored at -80° C.

For sample processing, plasma was diluted 1:10 in resuspension buffer. To 180 µl of the diluted plasma, 20 µl of the 10x precipitation buffer was added. This process of plasma dilution and precipitation was applied to all plasma standards, plasma samples and plasma QCs. After the addition of the 10x precipitation buffer, the sample was centrifuged at maximum speed at 4° C. for 5 min to remove non-specific plasma debris. 150 mL supernatant was then be transferred to a sample vial and then placed on an autosampler for a 100 mL injection.

The mobile phase (2L) was prepared with 13.6 g sodium acetate (50 mM), 2.1 g citric acid (5 mM) 36 mg EDTA (48 mM), 49.4 mg DTE (160 mM), and 2% methanol by volume in water. The pH was adjusted to 5.22. Resuspension buffer (20 mL) was made with 20 mL of PBS pH 7.4 (50 mM), 20 µL of 1 M DTE (1 mM), and 100 mL of 100 µM EDTA. The 10x precipitation buffer (25 mL) was made fresh with 2.88 mL phosphoric acid (1M), 9.39 g trichloroacetic acid (2 M) and 20 mL 1M DTE (1 mM).

Tetrahydrobiopterin (BH4), dihydrobiopterin (BH2), and Biopterin (B) were separated using reverse phase HPLC separation. BH4 was measured using electrochemical detection in which BH4 is oxidized by electrode 1 to quinonoid dihydrobiopterin (qBH2) and then reduced back to BH4 at electrode 2. The detector then uses the current generated by this reduction reaction to determine the concentration of BH4. BH2 and B can be measured in the same injection using fluorescence detection. Post column oxidation of BH2 using a conditioning guard cell at the optimum potential, oxidizes BH2 to Biopterin.

HPLC separation was carried out on an ACE C-18 (250 mm×4.6 mm) column, 5 µM, at a flow rate of 1.3 mL/min with a run time of 13 minutes. Electrochemical detection settings were E1: +100 mV (background current +500 nA to +600 nA) and E2: -300 mV (background current -50 nA to -60 nA). Post-column oxidation was set at 900 mV. Fluorescence detection settings were excitation wavelength: 350 nm and emission wavelength: 450 nm.

Linearity and range of the method were assessed based on the precision and accuracy of the standards in plasma and buffer. The standard curve concentration was established using at least 4-6 non-zero concentrations for each analyte. The concentration of the standards was 1, 2.5, 5, 7.5, 10, and 100 nM. The results showed a linear fit from 1 to 100 nM for BH4, BH2, and B with R<sup>2</sup> of >0.99.

Accuracy was determined by replicate analysis of quality control samples containing known amounts (2, 8, 25, and 50 nM) of the analyte and expressed as a percent accuracy. Precision is also calculated based on the data from the quality controls. Intra-assay precision and inter-assay precision were evaluated based on the CV %. On three separate experimental runs concentrations of each analyte were prepared in plasma and analyzed. In addition 10 nM of BH4, BH2, and B was "spiked" into human plasma samples to determine the accuracy and recovery. The measurements of BH4, BH2, and B at 8, 25, and 50 nM proved accurate within 112%-89% and demonstrated precision (CV %) of 2.5%-20%. Spike recovery experiments using 10 nM BH4, BH2, and B in clinical



samples of human plasma demonstrated recoveries between 70%-130%. The results demonstrate that the method is accurate and precise for samples with concentrations greater than 2 nM.

To check for the presence of endogenous interference in six different lots of plasma, 10 nM BH4, BH2, and B were spiked into six different lots of plasma and the determine accuracy and precision were determined for each plasma sample. Selectivity experiments show that the six individuals had endogenous baseline BH4 levels of between below quantifiable limit to 2.48 nM. Similarly, BH2 and B concentrations ranged from 0.02 to 10 nM. The recovery of the 10 nM spiked analytes ranged from 69%-87%. The variability (CV %) across the individual plasma samples and analytes when spiked at 10 nM ranged from 23%-37%. The variability of the endogenous levels of BH4, BH2 and B ranged from 0-9.96 nM. Together, the results indicate a trend suggesting matrix interference or loss during extraction, but do not indicate strong selectivity between individuals.

To measure matrix effect standard curves prepared in plasma or buffer were compared for accuracy (recovery), linearity and correlation. Comparison of the standards prepared in plasma versus standards prepared in buffer demonstrates a modest matrix effect and generally good correlation. All three analytes had excellent linear fits for plasma and buffer. BH4 and B did not demonstrate significant matrix effects across the concentration range. However, BH2 had less recovery at the highest standard concentration (100 nM). The quality control samples prepared in buffer and plasma demonstrated good accuracy. Overall, matrix effects seem minimal, with a trend toward less recovery in buffer as compared to plasma. Because BH4 and BH2 are readily oxidized, collected plasma and sample buffers should contain antioxidants and have low pH when possible.

To test the ability to accurately dilute a plasma and buffer sample spiked with 250 nM of BH4, BH2 and B, plasma was diluted using blank plasma in a 3-fold dilution series. The diluted samples were analyzed and compared to the nominal value after the dilution factor was applied. The dilution of high concentrations of BH4, BH2, and B can be accurately made. For BH4 the observed concentrations following dilution were between 83%-104% accurate for concentrations between 83.33 nM and 3.07 nM. BH2 was 74%-80% accurate across the quantitative range (83 nM-3 nM). B was 119%-113% accurate across the quantitative range (83 nM-3 nM). Therefore, a sample that is above the quantitative limit can be diluted accurately.

Four concentrations of analytes (2, 8, 25 and 50 nM) were prepared in plasma and frozen for a minimum of 24 hours for one cycle and a minimum of 12 hours for other cycles for a minimum of three cycles. Samples were thawed unassisted at room temperature in between frozen periods. The accuracy and variability after each and all free-thaw cycles was assessed to establish the maximal number of cycles a sample could undergo. The BH4-, BH2-, and B-containing samples can undergo up to 3 freeze-thaw cycles without significant change in accuracy or precision of the measurement. Plasma samples with 8 nM-50 nM BH4 are 121%-91% accurate and CV % less than 10%. Similarly BH2 measurements were 77%-88% accurate across the quantitative range of the assay. B measurements were 98%-99% accurate across the quantitative range with precision (CV %) of 5%-8%. The 2 nM sample of BH4, BH2, and B did not prove accurate or precise following repeated freeze-thaw. Therefore, standards, quality controls and study samples may be frozen and thawed up to 3 times.

Because the analytes are sensitive to oxidation we examined long-term frozen stability to mimic expected storage conditions. Four concentration levels (2, 8, 50, and 100 nM) of BH4, BH2, and B were prepared in plasma and stored at -70° C. for 8 weeks. Stability samples were assayed fresh and at weeks 3, 5, 6, and 8. BH4 and B had good long term frozen stability. BH2 demonstrated reduced sample concentration after prolonged storage. Over the 8 weeks of storage, plasma samples with BH4 were 93%-94% accurate and had CV % between 31%-0.21%, with the most variation seen at the 2 nM concentration. BH2 measurements were 63%-85% accurate across the concentrations tested with reduced accuracy at the 2 nM and 100 nM concentrations. The precision (CV %) ranged from 37% to 18% for these samples. B measurements were 88%-101% accurate across the concentrations tested with precision (CV %) of 23%-0.14%, with the highest variability at the 2 nM concentration. Together, this data supports the recommendation to store samples for up to 8 weeks without appreciable loss of analyte concentration. BH2 seems to be the most susceptible to degradation (oxidation).

To measure the stability of BH4, BH2, and B in the autosampler, 8 nM of each analyte in reconstitution solvent stayed on the autosampler for 0.25, 4, and 11 hours. The accuracy and precision of the measurements were compared. The observed BH4 measurement was accurate within 5% of theoretical at each time point with accuracy and precision across all three measurements of 102% and 0.054% respectively. The measurement of BH2 had decreasing accuracy and increasing variability after 4 hours. After 11 hours on the autosampler about 50% of the BH2 was measured. This indicates poor autosampler stability in run buffer. The measurement of B remained accurate within 125% of theoretical after 11 hours. Therefore, run times of no more than 4 hours are recommended.

To determine injection carry-over, an extracted baseline plasma sample was inserted after the highest standard concentration 100 nM. This was done to mimic the possibility of overestimating the concentration of analyte in a low concentration sample due to carry-over. The injection carryover of BH4, BH2, and B is minimal and does not account for more than 1% of the peak area of the 100 nM upper limit of quantitation. The injection carryover accounts for approximately 5%-20% of the lower limit of quantitation, based on the average peak area obtained from the low quality control (2 nM). Therefore, preferably the samples should be ordered from lowest to high (i.e., pre-dose first, followed by post-dose samples) and additional washes to clean the column periodically during a run preferably will be made to minimize potential carryover.

A qualified method which was robust, specific, accurate and precise was developed. This method is appropriate to quantify the levels of BH4, BH2 and B in plasma for pharmacokinetic and drug studies.

All patents, publications and references cited herein are hereby fully incorporated by reference. In case of conflict between the present disclosure and incorporated patents, publications and references, the present disclosure will control.

What is claimed is:

1. A method of orally administering tetrahydrobiopterin (BH4), comprising separately administering to a human in need thereof (i) a therapeutically effective amount of BH4 or a pharmaceutically acceptable salt thereof and (ii) food, wherein the BH4 or pharmaceutically acceptable salt thereof is administered at the same time as or within 30 minutes after the food.

2. The method of claim 1, further comprising informing the human that Cmax and AUC are increased by approximately

## US RE43,797 E

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30% when the BH4 or pharmaceutically acceptable salt thereof is administered with a high-fat, high-calorie meal compared to when the BH4 or pharmaceutically acceptable salt thereof is administered under fasting conditions.

3. The method of claim 1, wherein said BH4 is a crystalline polymorph, as a hydrochloride salt, that exhibits an X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values(A): 8.7 (vs), 5.63 (m), 4.76 (m), 4.40 (m), 4.00 (s), 3.23 (s), 3.11 (vs), preferably 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (w), 2.69 (w), 2.59 (w), and 2.44 (w).

4. The method of claim 1, wherein the BH4 or pharmaceutically acceptable salt thereof is administered at a daily dose of at least 5 mg/kg.

5. The method of claim 1, wherein the BH4 or pharmaceutically acceptable salt thereof is administered at a daily dose of at least 20 mg/kg.

6. The method of claim 1, wherein the human has been diagnosed with hyperphenylalaninemia, a neuropsychiatric disorder, a cardiovascular disease, anemia, or a combination thereof.

7. The method of claim 1, further comprising informing said human that absorption of said BH4 or pharmaceutically acceptable salt thereof is increased when it is ingested with food, compared to when ingested without food.

8. The method of claim 1, wherein said human suffers from one or more disorders selected from the group consisting of phenylketonuria, hyperphenylalaninemia, BH4 deficiency, dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, or dihydropteridine reductase (DHPR) deficiency, Parkinson's disease, dystonia, pain, fatigue, depression, an affective disorder, schizophrenia, stroke, migraine headaches, Alzheimer's disease, schizophreniform disorder, schizoaffective disorder, brief psychotic disorder, delusional disorder, shared psychotic disorder, psychotic disorder due to a general medical condition, substance-induced psychotic disorder, other psychotic disorders, tardive dyskinesia, chronic fatigue syndrome, acute or chronic depression, chronic stress syndrome, fibromyalgia, attention deficit hyperactivity disorder, bipolar disease, and autism.

9. The method of claim 1, wherein the food comprises a high fat, high calorie meal.

10. The method of claim 1 or claim 8, further comprising dissolving the BH4 or pharmaceutically acceptable salt thereof in a liquid prior to administering to the human.

11. The method of claim 1 or claim 8, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as an intact tablet.

12. The method of claim 1 or claim 8, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as a solid dosage form.

13. The method of claim 1, wherein the BH4 or pharmaceutically acceptable salt thereof is administered once per day.

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14. The method of claim 13, wherein the human suffers from hyperphenylalaninemia.

15. The method of claim 13, wherein the human suffers from one or more disorders selected from the group consisting of BH4 deficiency, dopa-responsive dystonia (DRD), sepiapterin reductase (SR) deficiency, and dihydropteridine reductase (DHPR) deficiency.

16. The method of claim 13, wherein the human suffers from one or more disorders selected from the group consisting of Parkinson's disease, dystonia, pain, fatigue, depression, an affective disorder, schizophrenia, stroke, migraine headaches, Alzheimer's disease, schizophreniform disorder, schizoaffective disorder, brief psychotic disorder, delusional disorder, shared psychotic disorder, psychotic disorder due to a general medical condition, substance-induced psychotic disorder, other psychotic disorders, tardive dyskinesia, chronic fatigue syndrome, acute or chronic depression, chronic stress syndrome, fibromyalgia, and bipolar disease.

17. The method of claim 13, wherein the human suffers from attention deficit hyperactivity disorder or autism.

18. The method of claim 13, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as a solid dosage form.

19. The method of claim 13, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as an intact tablet.

20. The method of claim 13, further comprising dissolving the BH4 or pharmaceutically acceptable salt thereof in a liquid prior to administering to the human.

21. A method of orally administering tetrahydrobiopterin (BH4) to a human suffering from hyperphenylalaninemia, comprising separately administering to said human (i) a therapeutically effective amount of BH4 or pharmaceutically acceptable salt thereof, once per day, and (ii) food, wherein the BH4 or pharmaceutically acceptable salt thereof is administered at the same time as or within 30 minutes after the food.

22. The method of claim 21, wherein the human suffers from phenylketonuria.

23. The method of claim 21, wherein the therapeutically effective amount of BH4 or pharmaceutically acceptable salt thereof is at least 5 mg/kg.

24. The method of claim 21, wherein the therapeutically effective amount of BH4 or pharmaceutically acceptable salt thereof is 1 mg/kg to 30 mg/kg.

25. The method of claim 21, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as a solid dosage form.

26. The method of claim 21, wherein the BH4 or pharmaceutically acceptable salt thereof is administered as an intact tablet.

27. The method of claim 21, further comprising dissolving the BH4 or pharmaceutically acceptable salt thereof in a liquid prior to administering to the human.

28. The method of claim 21, wherein the food comprises a high fat, high calorie meal.

\* \* \* \* \*

# EXHIBIT G

US008318745B2

(12) **United States Patent**  
**Moser et al.**

(10) **Patent No.:** **US 8,318,745 B2**  
(45) **Date of Patent:** **\*Nov. 27, 2012**

(54) **CRYSTALLINE FORMS OF  
(6R)-L-ERYTHRO-TETRAHYDROBIOPTERIN  
DIHYDROCHLORIDE**

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Egger**, Kempthal (CH); **Fritz Blatter**,  
Reinach (CH)

(73) Assignee: **Merck & CIE**, Schaffhausen (CH)

(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 0 days.

This patent is subject to a terminal dis-  
claimer.

(21) Appl. No.: **12/629,963**

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(65) **Prior Publication Data**

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(63) Continuation of application No. 10/990,316, filed on  
Nov. 17, 2004, now Pat. No. 7,727,987.

(60) Provisional application No. 60/520,377, filed on Nov.  
17, 2003.

(51) **Int. Cl.**  
**C07D 475/04** (2006.01)  
**A61K 31/4985** (2006.01)  
**A61P 25/16** (2006.01)

(52) **U.S. Cl.** ..... **514/252.12**; 544/258

(58) **Field of Classification Search** ..... 544/258  
See application file for complete search history.

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*Assistant Examiner* — Cecilia M Jaisle

(74) *Attorney, Agent, or Firm* — Millen, White, Zelano &  
Branigan, P.C.

(57) **ABSTRACT**

Crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihy-  
drochloride, hydrates and solvates and processes for their  
preparation are provided. These crystal forms are either inter-  
mediates for the preparation of stable polymorphic form B or  
are suitable for solid formulations.

**22 Claims, 15 Drawing Sheets**

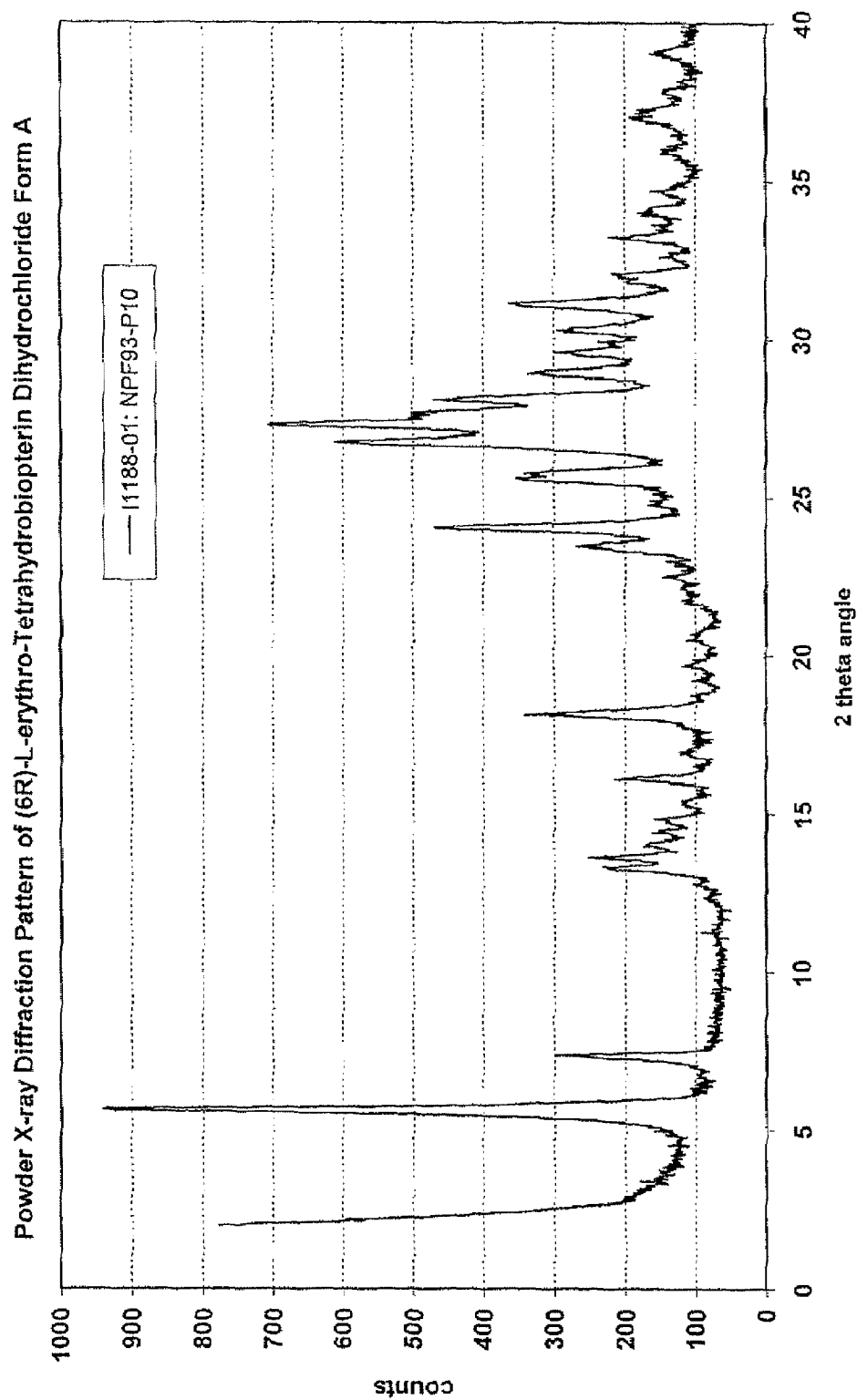
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Figure 1



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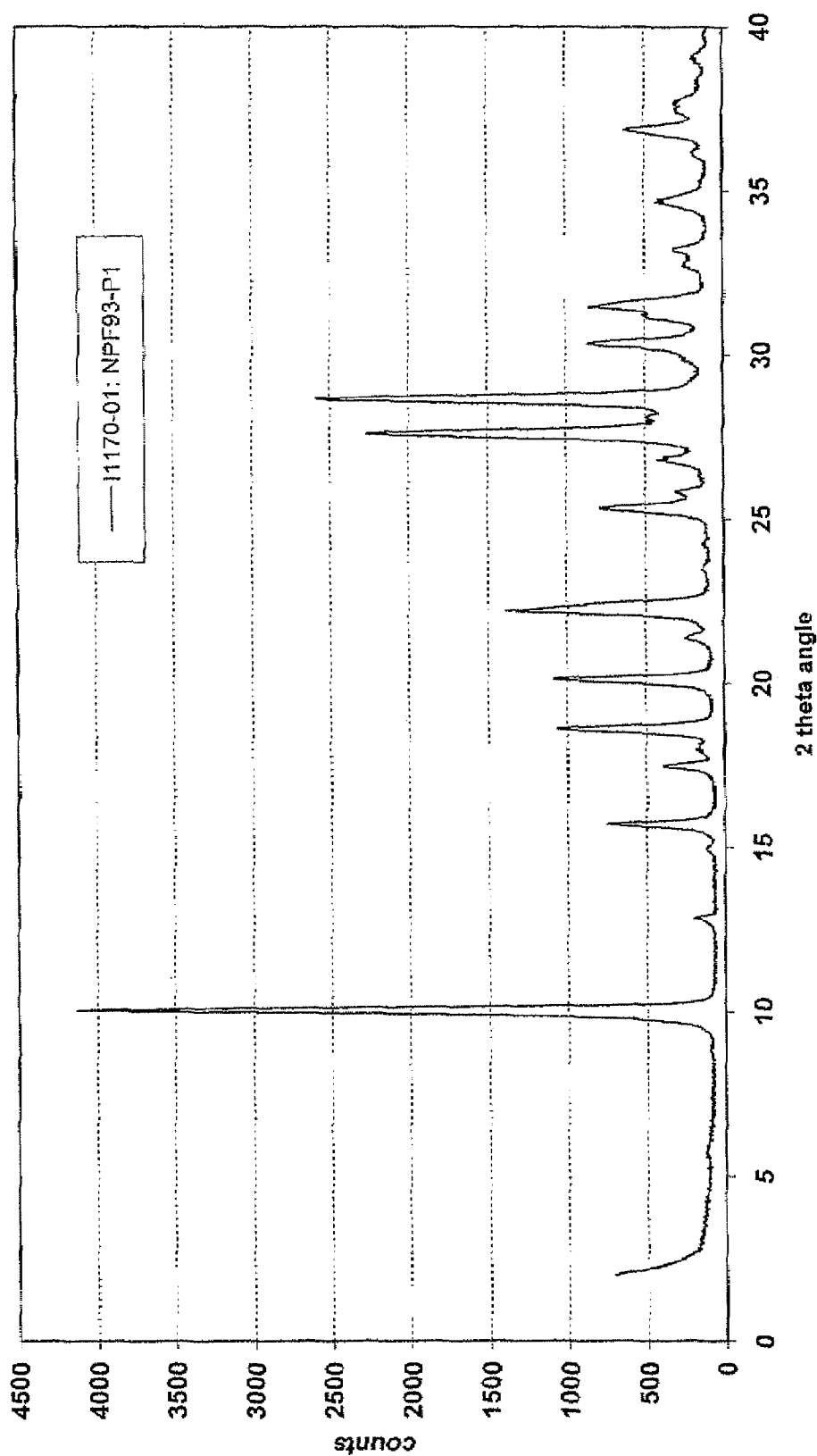
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Figure 2

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form B





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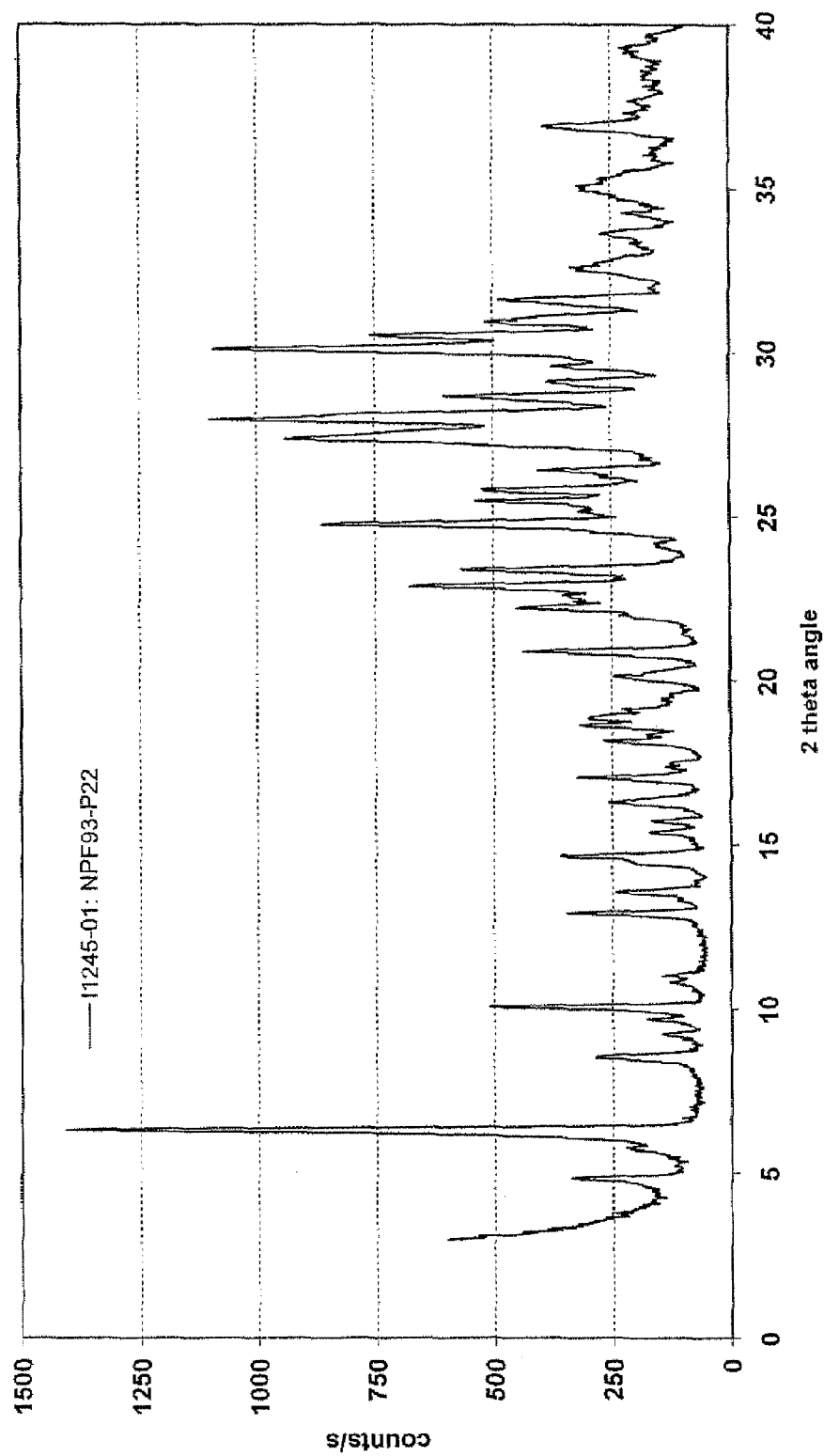
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Figure 3

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochlorid Form C



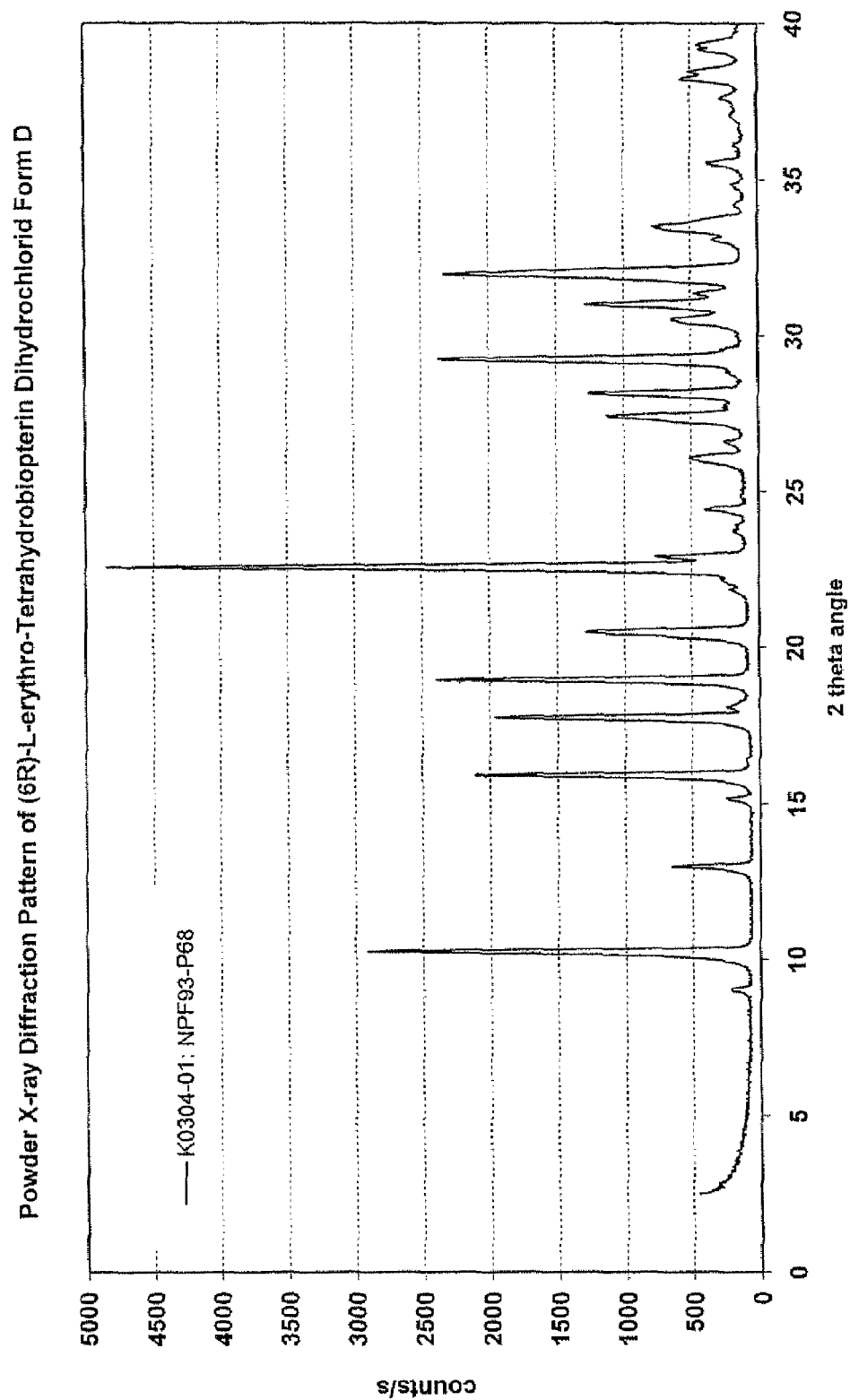
U.S. Patent

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Figure 4



U.S. Patent

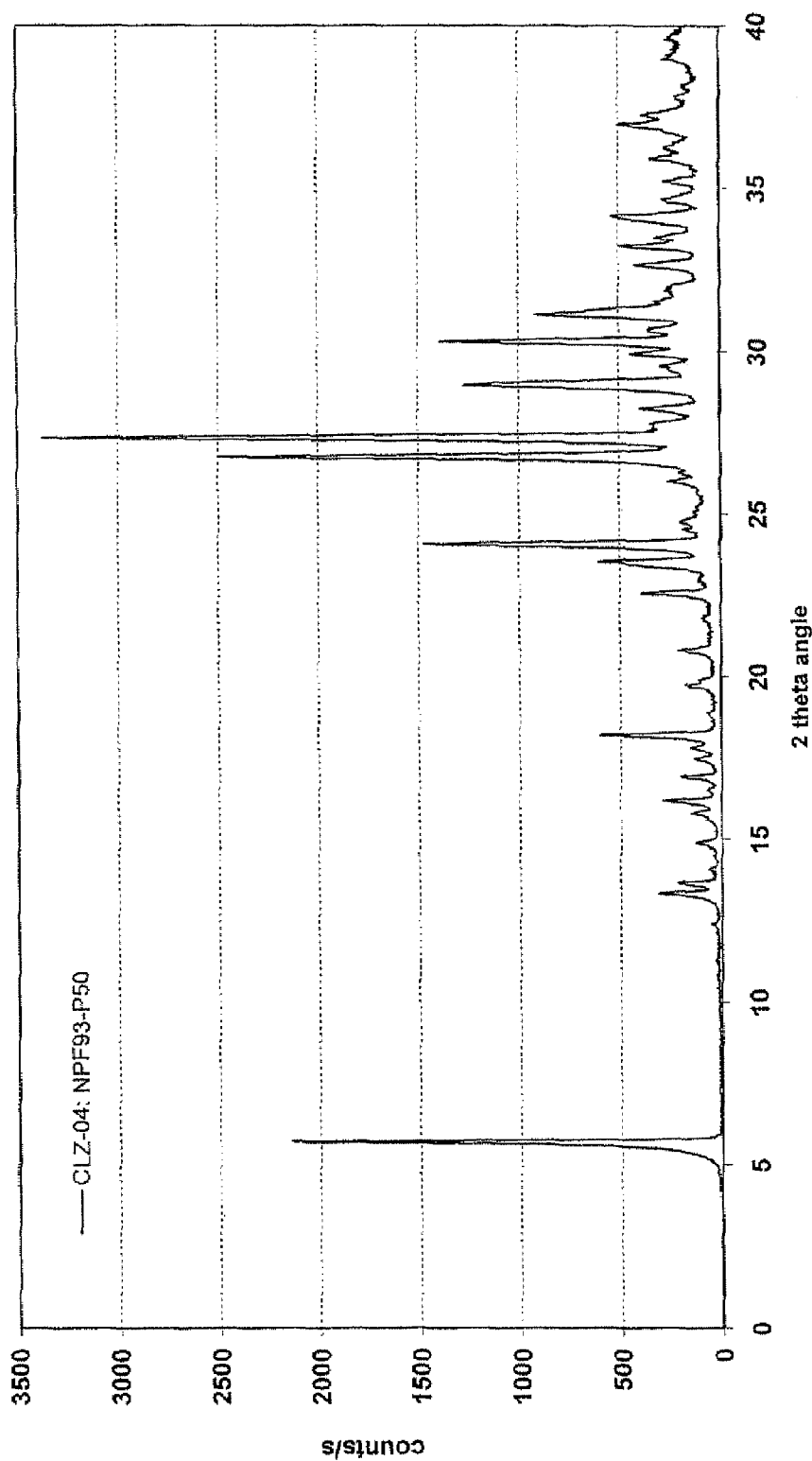
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Figure 5

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form E



U.S. Patent

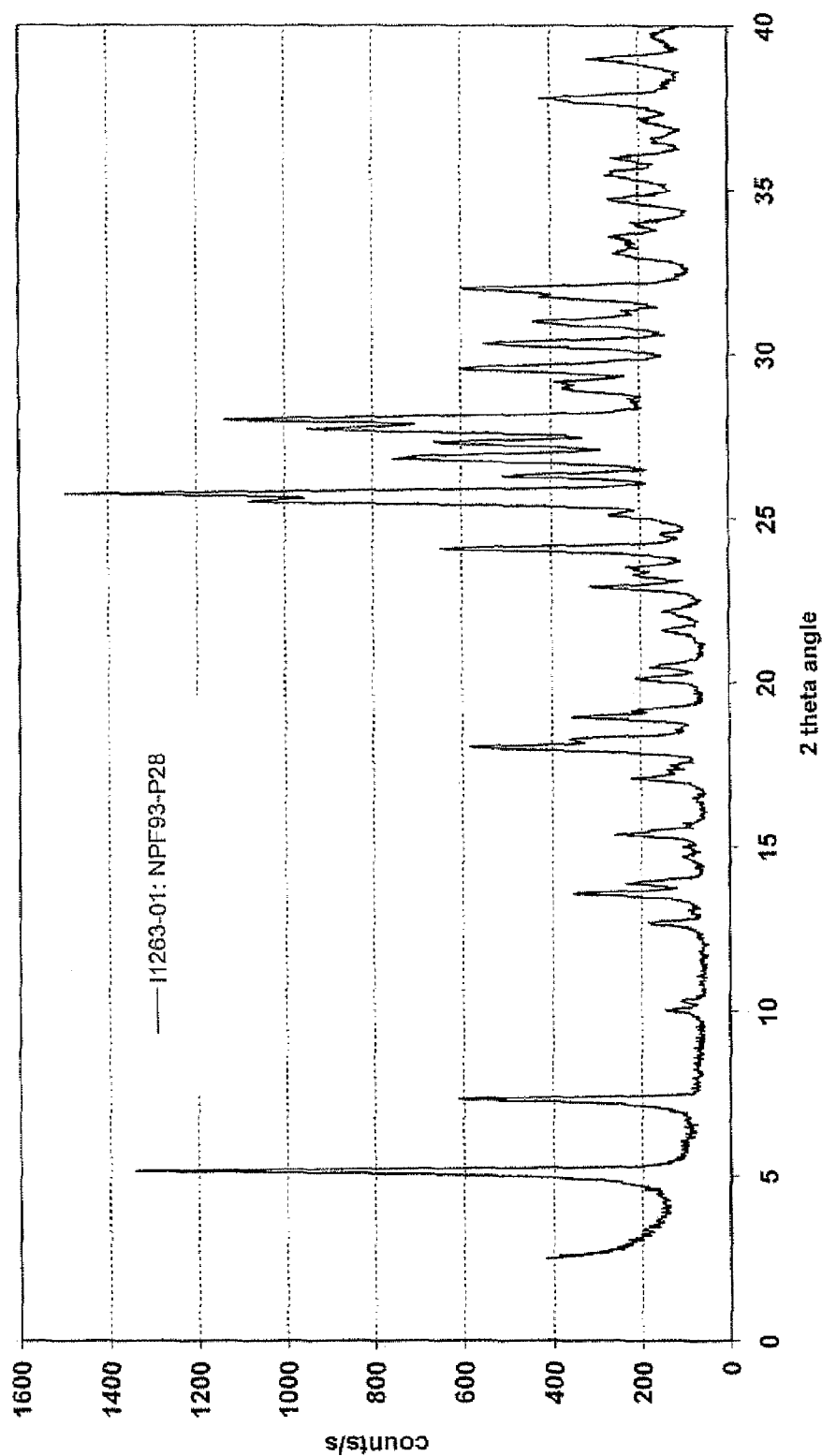
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Figure 6

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form F



U.S. Patent

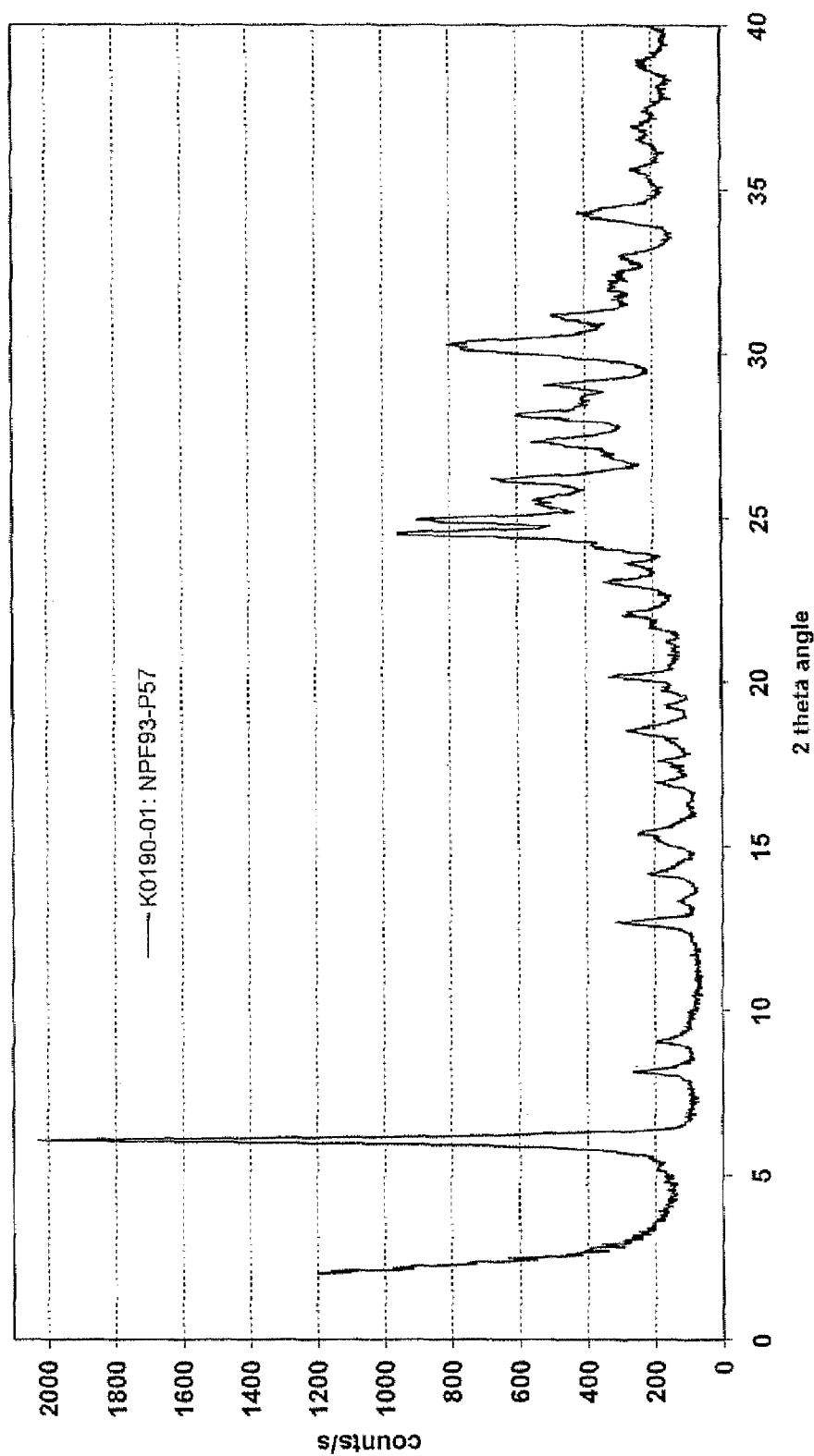
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Figure 7

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form G



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Figure 8

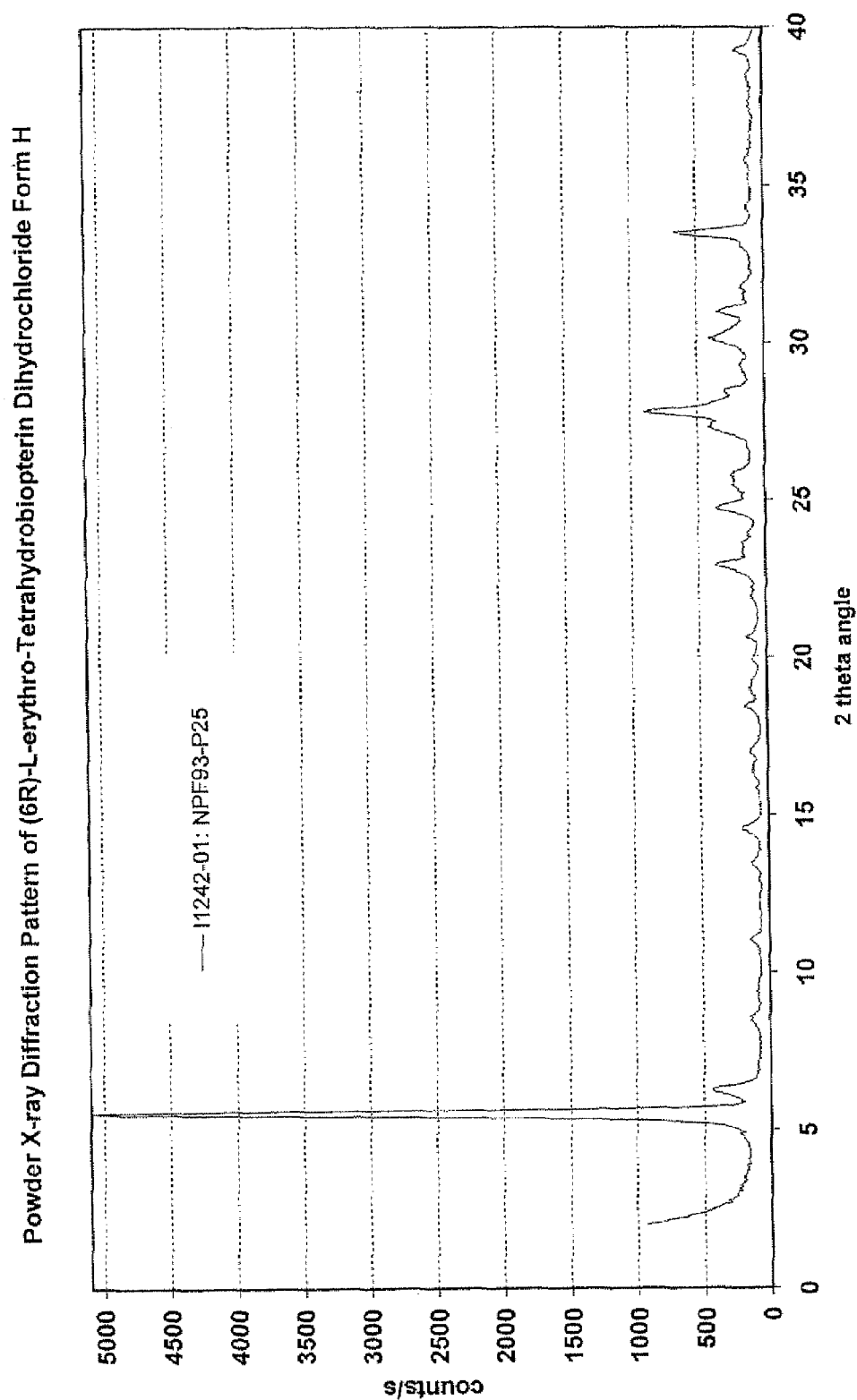
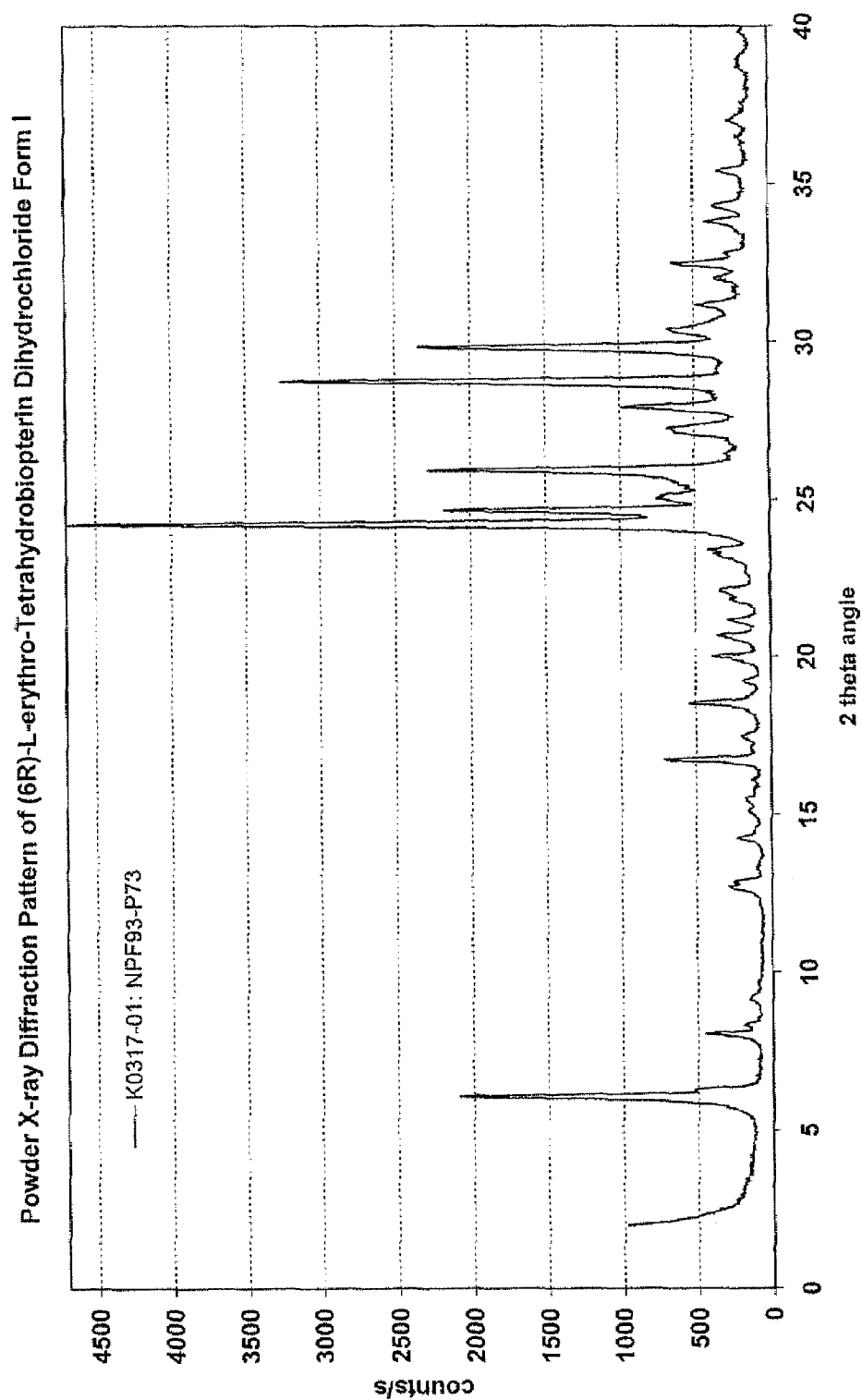




Figure 9



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Figure 10

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form J

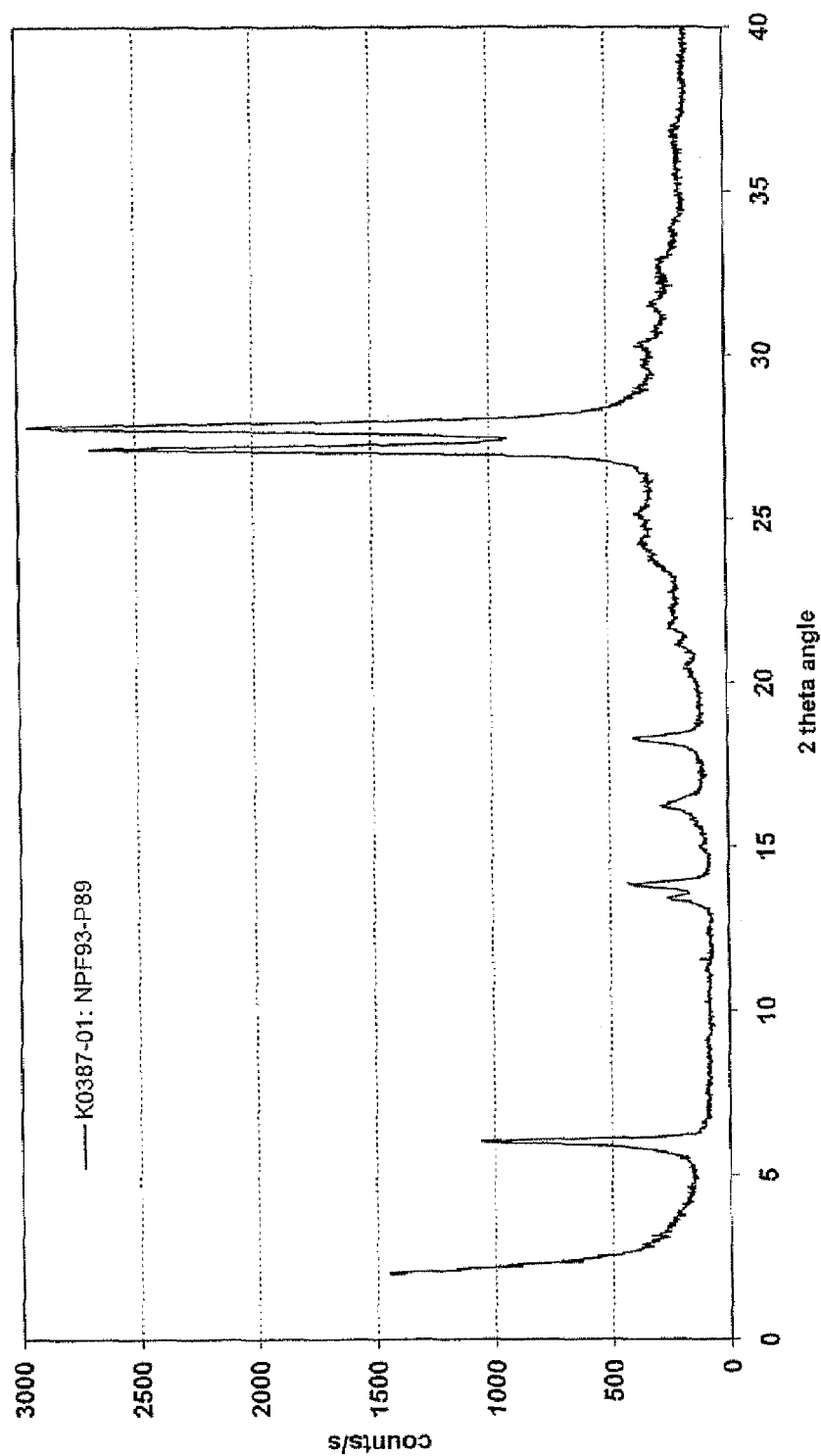


Figure 11

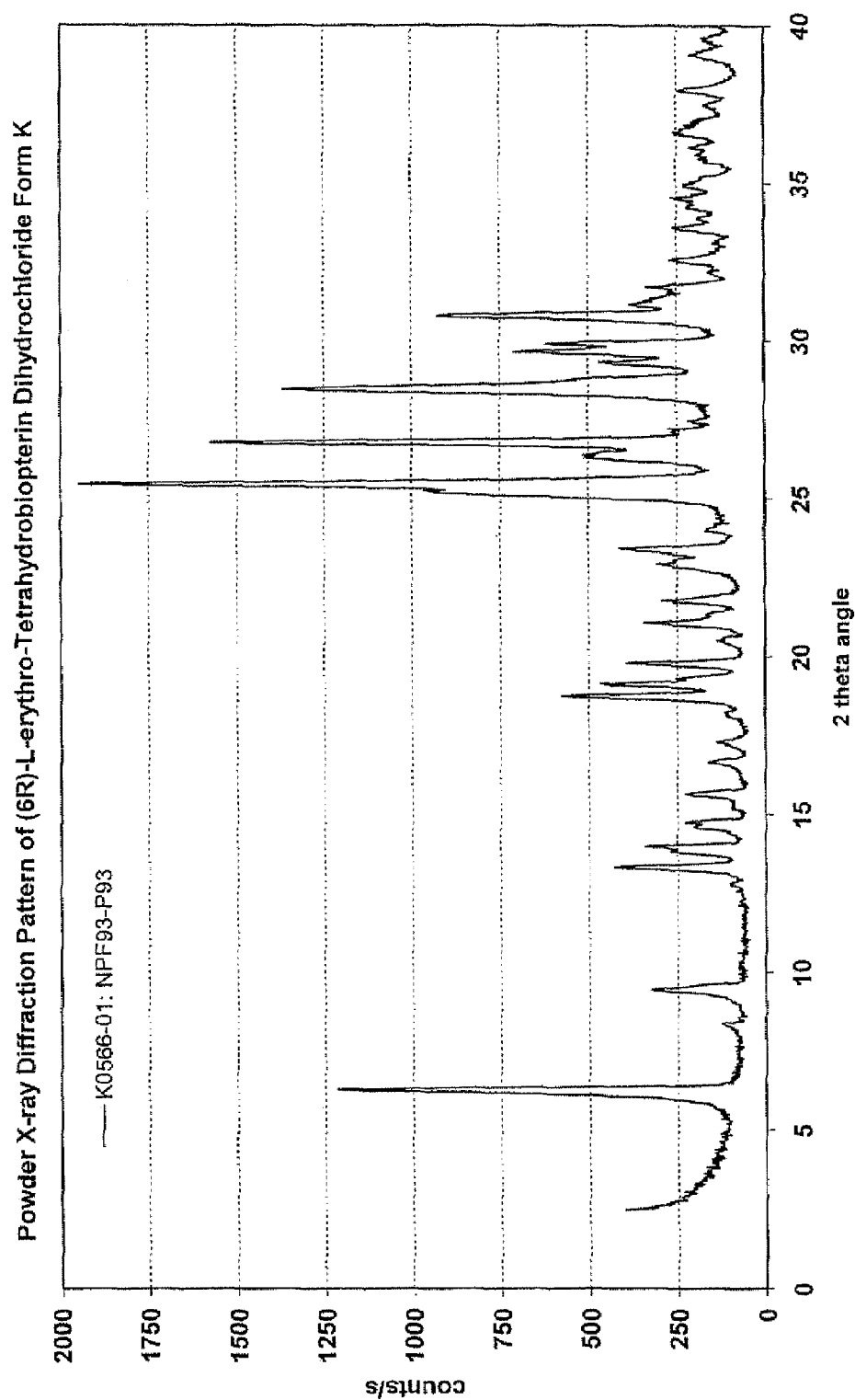
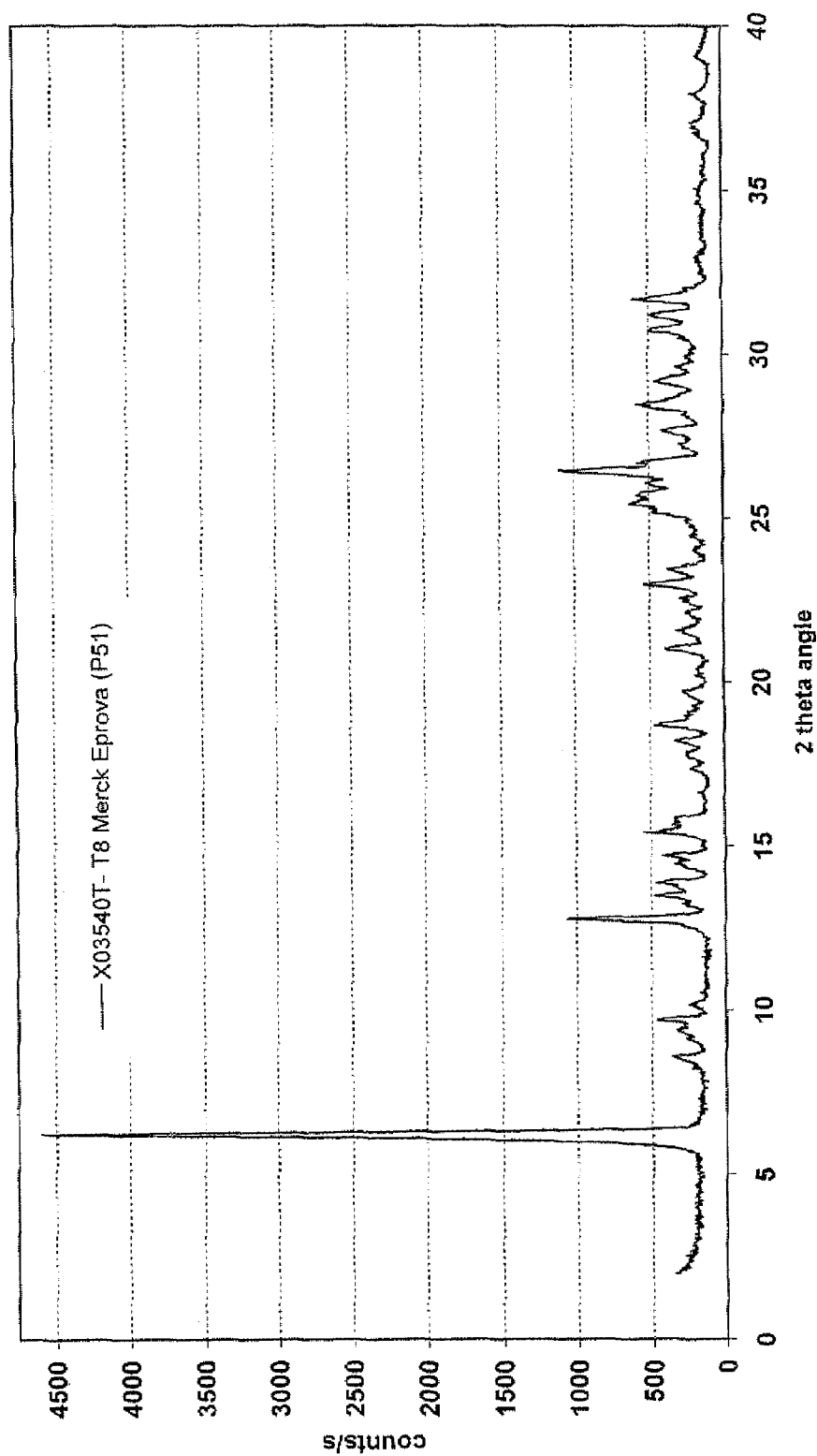


Figure 12

Powder X-ray Diffraction Pattern of (6R)-L-erythro-Tetrahydrobiopterin Dihydrochloride Form L



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Figure 13

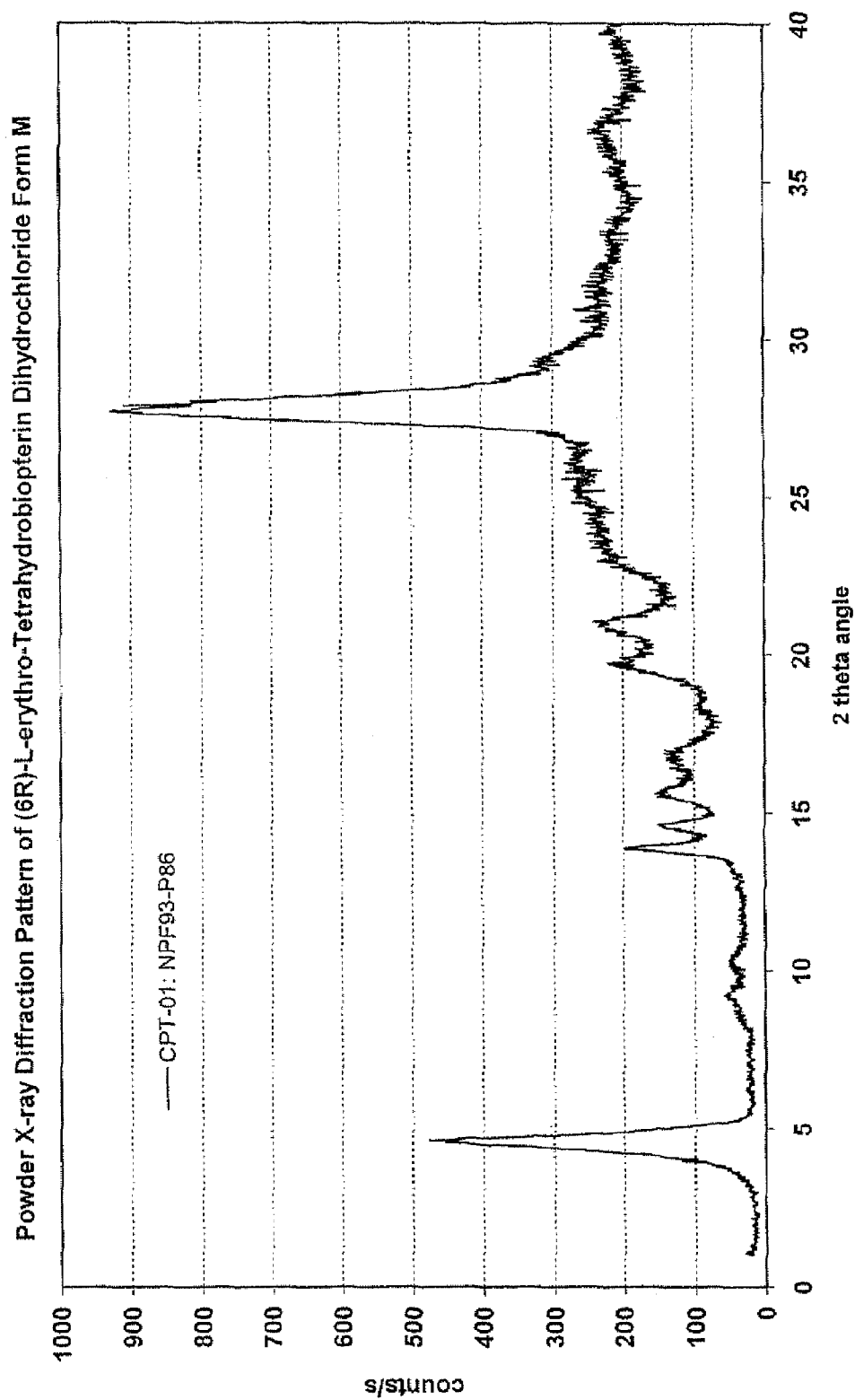


Figure 14

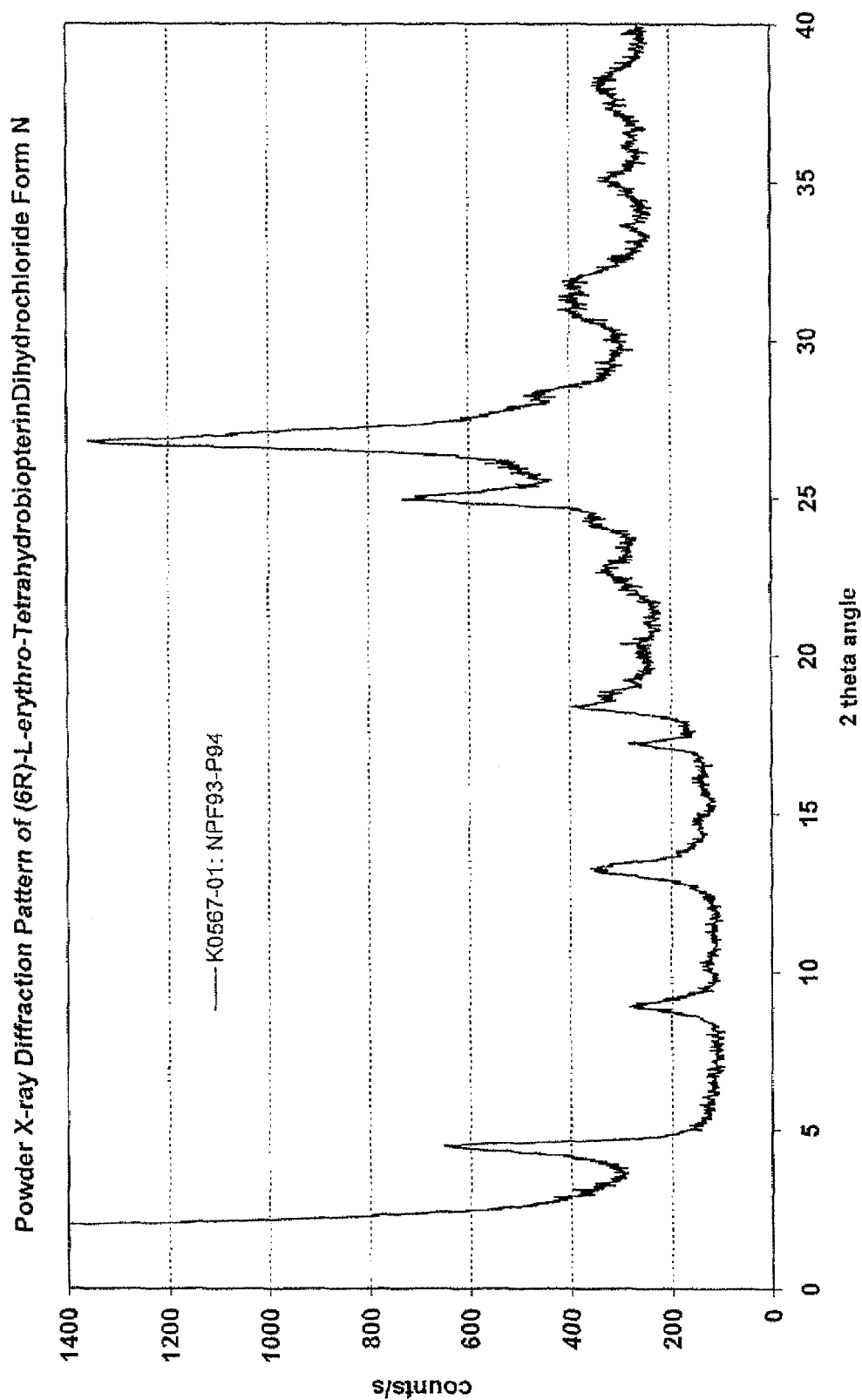
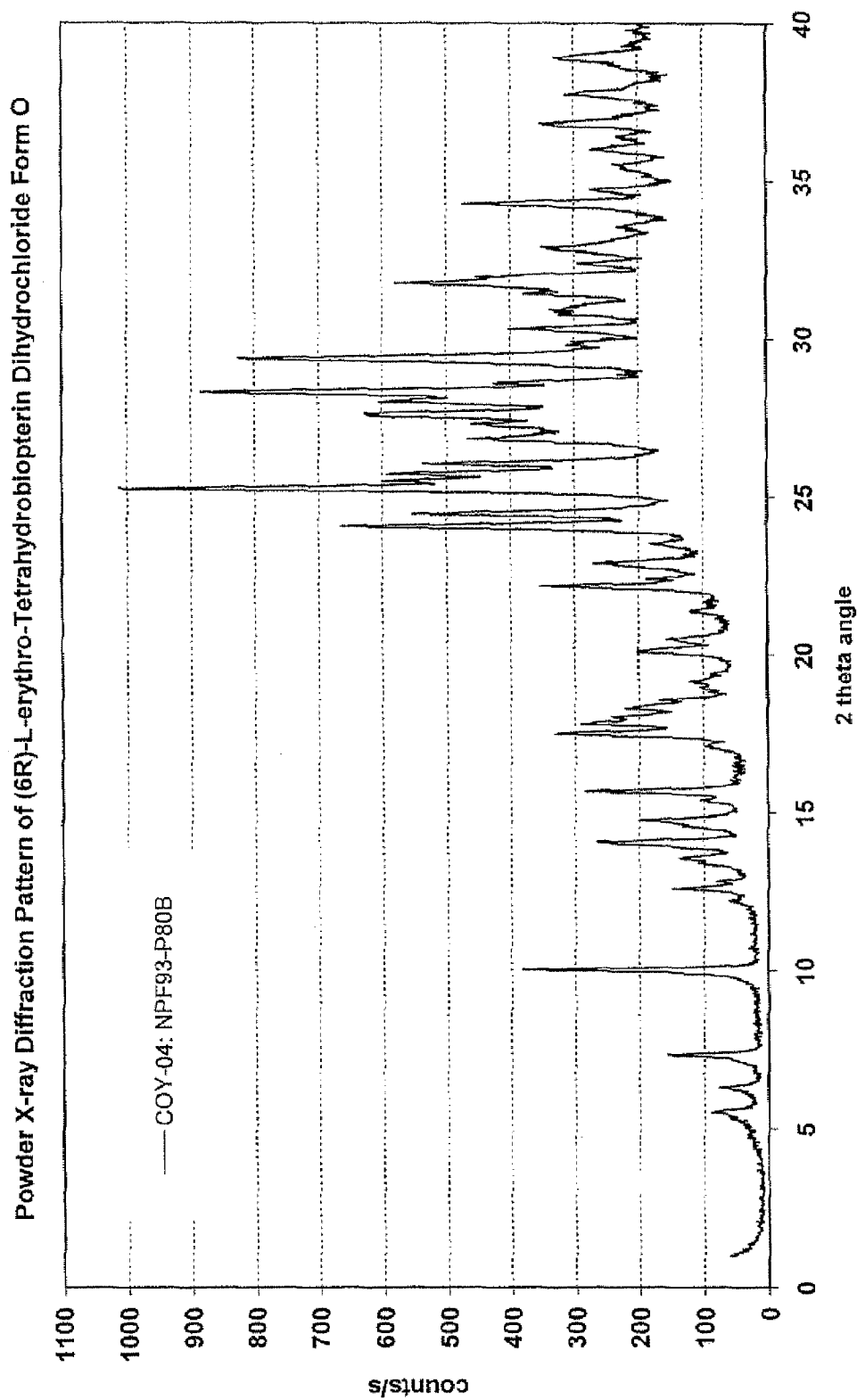




Figure 15



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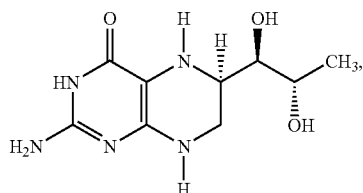
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# CRYSTALLINE FORMS OF (6R)-L-ERYTHRO-TETRAHYDROBIOPTERIN DIHYDROCHLORIDE

This application is a continuation of U.S. application Ser. No. 10/990,316, filed Nov. 17, 2004 now U.S. Pat. No. 7,727, 987 and claims the benefit of the filing date of U.S. Provisional Application Ser. No. 60/520,377, filed Nov. 17, 2003 which is incorporated by reference herein.

The present invention relates to crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and hydrates and solvates thereof. This invention also relates to processes for preparing the crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and hydrates and solvates thereof. This invention also relates to compositions comprising selected and stable crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride or a hydrate thereof and a pharmaceutically acceptable carrier.

It is known that the biosynthesis of the neurotransmitter catecholamines from phenylalanine requires tetrahydrobiopterin cofactor, (6R)-2-amino-4-oxo-6-[(1R,2S)-1,2-dihydroxypropyl]-5,6,7,8-tetrahydropteridine according to formula (I),



at the monooxygenation step of phenylalanine and tyrosine. It is supposed that the catecholamine biosynthesis is regulated in a great extent by tetrahydrobiopterin cofactor, and that a decrease of the cofactor in central nerve systems causes several neurological disorders such as parkinsonism and atypical phenylketonuria. The compound of formula I is therefore an effective therapeutic agent for treatment of said disorders in mammals in need thereof.

The compound of formula I is difficult to handle and it is therefore produced and offered as its dihydrochloride salt (Schircks Laboratories, CH-8645 Jona, Switzerland) even in ampoules sealed under nitrogen to prevent degradation of the substance due to its hygroscopic nature and sensitivity to oxidation. U.S. Pat. No. 4,649,197 discloses that separation of (6R)- and 6(S)-L-erythro-tetrahydrobiopterin dihydrochloride into its diastereomers is difficult due to the poor crystallinity of 6(R,S)-L-erythro-tetrahydrobiopterin dihydrochloride. In EP-A1-0 079 574 is described the preparation of tetrahydrobiopterin, where a solid tetrahydrobiopterin dihydrochloride is obtained as an intermediate. S. Matsuura et al. describes in Chemistry Letters 1984, pages 735-738 and Heterocycles, Vol. 23, No. 12, 1985 pages 3115-3120 6(R)-tetrahydrobiopterin dihydrochloride as a crystalline solid in form of colourless needles, which are characterized by X-ray analysis disclosed in J. Biochem. 98, 1341-1348 (1985). An optical rotation of 6.81° was found the crystalline product, which is quite similar to the optical rotation of 6.51° reported for a crystalline solid in form of white crystals in example 6 of EP-A2-0 191 335.

Results obtained during investigation and development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride development revealed that the known crystalline solids can be

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designated as form B, for which was found a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

8.7 (vs), 6.9 (w), 5.90 (vw), 5.63 (m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w), 2.44 (w). A characteristic X-ray powder diffraction pattern is exhibited in FIG. 2.

Here and in the following the abbreviations in brackets mean: (vs)=very strong intensity; (s)=strong intensity; (m)=medium intensity; (w)=weak intensity; and (vw)=very weak intensity.

Polymorph B is a slightly hygroscopic anhydrate with the highest thermodynamic stability above about 20° C. Furthermore, form B can be easily processed and handled due to its thermal stability, possibility for preparation by targeted conditions, its suitable morphology and particle size. Melting point is near 260° C. ( $\Delta H_f > 140$  J/g), but no clear melting point can be detected due to decomposition prior and during melting. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application, which are prepared at elevated temperatures. Polymorph B can be obtained as a fine powder with a particle size that may range from 0.2  $\mu$ m to 500  $\mu$ m.

However, there is a need for other stable forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride with satisfactory chemical and physical stability for a safe handling during manufacture and formulation as well as providing a high storage stability in its pure form or in formulations. In addition, there is a strong need for processes to produce polymorph B and other crystalline forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride on a large scale in a controlled manner.

Results obtained during development of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride indicated that the compound may exist in different crystalline forms, including polymorphic forms and solvates. The continued interest in this area requires an efficient and reliable method for the preparation of the individual crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and controlled crystallization conditions to provide crystal forms, that are preferably stable and easy to handle and to process in the manufacture and preparation of formulations, and that provide a high storage stability in substance form or as formulated product, or which provide less stable forms suitable as intermediates for controlled crystallisation for the manufacture of stable forms.

## 1. Polymorphic Forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride

Polymorphic forms A, B, F, J and K are anhydrides, which absorb up to about 3% by weight of water when exposed to open air humidity at ambient temperature.

A first object of the invention is crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å): 15.5 (vs), 12.0 (m), 4.89 (m), 3.70 (s), 3.33 (s), 3.26 (s), and 3.18 (m); hereinafter designated as form A.

In a more preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.5 (vs), 12.0 (m), 6.7 (m), 6.5 (m), 6.3 (w), 6.1 (w), 5.96 (w), 5.49 (m), 4.89 (m), 3.79 (m), 3.70 (s), 3.48 (m), 3.45 (m),

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3.33 (s), 3.26 (s), 3.22 (m), 3.18 (m), 3.08 (m), 3.02 (w), 2.95 (w), 2.87 (m), 2.79 (w), 2.70 (w);

hereinafter designated as form A.

In another preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits characteristic Raman bands, expressed in wave numbers ( $\text{cm}^{-1}$ ) at:

2934 (w), 2880 (w), 1692 (s), 1683 (m), 1577 (w), 1462 (m), 1360 (w), 1237 (w), 1108 (w), 1005 (vw), 881 (vw), 813 (vw), 717 (m), 687 (m), 673 (m), 659 (m), 550 (w), 530 (w), 492 (m), 371 (m), 258 (w), 207 (w), 101 (s), 87 (s)  $\text{cm}^{-1}$ ,

hereinafter designated as form A.

In still another preferred embodiment, the present invention comprises a crystalline polymorph A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 1.

The polymorph A is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph A is a hygroscopic anhydrate which is a meta-stable form with respect to form B; however, it is stable over several months at ambient conditions if kept in a tightly sealed container. Form A is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form A can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Still another object of the invention is crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

17.1 (vs), 4.92 (m), 4.68 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.21 (m), and 3.19 (m),

hereinafter designated as form F.

In a more preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

17.1 (vs), 12.1 (w), 8.6 (w), 7.0 (w), 6.5 (w), 6.4 (w), 5.92 (w), 5.72 (w), 5.11 (w), 4.92 (m), 4.86 (w), 4.68 (m), 4.41 (w), 4.12 (w), 3.88 (w), 3.83 (w), 3.70 (m), 3.64 (w), 3.55 (m), 3.49 (s), 3.46 (vs), 3.39 (s), 3.33 (m), 3.31 (m), 3.27 (m), 3.21 (m), 3.19 (m), 3.09 (m), 3.02 (m), and 2.96 (m),

hereinafter designated as form F.

In still another preferred embodiment, the present invention comprises a crystalline polymorph F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 6.

The polymorph F is slightly hygroscopic and adsorbs water to a content of about 3 percent by weight, which is continuously released between 50° C. and 200° C., when heated at a rate of 10° C./minute. The polymorph F is a meta-stable form and a hygroscopic anhydrate, which is more stable than form A at ambient lower temperatures and less stable than form B at higher temperatures and form F is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form F can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Still another object of the invention is a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

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14.6 (m), 3.29 (vs), and 3.21 (vs), hereinafter designated as form J.

In a more preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

14.6 (m), 6.6 (w), 6.4 (w), 5.47 (w), 4.84 (w), 3.29 (vs), and 3.21 (vs),

hereinafter designated as form J.

In still another preferred embodiment, the present invention comprises a crystalline polymorph J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 10.

The polymorph J is slightly hygroscopic and adsorbs water when handled at air humidity. The polymorph J is a meta-stable form and a hygroscopic anhydrate, and it can be transformed back into form E from which it is obtained upon exposure to high relative humidity conditions such as above 75% relative humidity. Form J is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form J can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Still another object of the invention is a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

14.0 (s), 6.6 (w), 4.73 (m), 4.64 (m), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m),

hereinafter designated as form K.

In a more preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

14.0 (s), 9.4 (w), 6.6 (w), 6.4 (w), 6.3 (w), 6.1 (w), 6.0 (w), 5.66 (w), 5.33 (w), 5.13 (vw), 4.73 (m), 4.64 (m), 4.48 (w), 4.32 (vw), 4.22 (w), 4.08 (w), 3.88 (w), 3.79 (w), 3.54 (m), 3.49 (vs), 3.39 (m), 3.33 (vs), 3.13 (s), 3.10 (m), 3.05 (m), 3.01 (m), 2.99 (m), and 2.90 (m),

hereinafter designated as form K.

In still another preferred embodiment, the present invention comprises a crystalline polymorph K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 11.

The polymorph K is slightly hygroscopic and adsorbs water to a content of about 2.0 percent by weight, which is continuously released between 50° C. and 100° C., when heated at a rate of 10° C./minute. The polymorph K is a meta-stable form and a hygroscopic anhydrate, which is less stable than form B at higher temperatures and form K is especially suitable as intermediate and starting material to produce stable polymorph forms, in particular form B. Polymorph form K can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

## 2. Hydrate Forms of

(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

(6R)-L-erythro-tetrahydrobiopterin dihydrochloride forms crystalline hydrate forms C, D, E, H and O, depending from the preparation method.

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Still another object of the invention is a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

13.9 (vs), 8.8 (m), 6.8 (m), 6.05 (m), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 2.97 (m), and 2.93 (vs),

hereinafter designated as form C.

In a more preferred embodiment, the present invention comprises a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

18.2 (m), 15.4 (w), 13.9 (vs), 10.4 (w), 9.6 (w), 9.1 (w), 8.8 (m), 8.2 (w), 8.0 (w), 6.8 (m), 6.5 (w), 6.05 (m), 5.77 (w), 5.64 (w), 5.44 (w), 5.19 (w), 4.89 (w), 4.76 (w), 4.70 (w), 4.41 (w), 4.25 (m), 4.00 (m), 3.88 (m), 3.80 (m), 3.59 (s), 3.50 (m), 3.44 (m), 3.37 (m), 3.26 (s), 3.19 (vs), 3.17 (s), 3.11 (m), 3.06 (m), 3.02 (m), 2.97 (vs), 2.93 (m), 2.89 (m), 2.83 (m), and 2.43 (m),

hereinafter designated as form C.

In still another preferred embodiment, the present invention comprises a crystalline hydrate C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 3.

The hydrate form C is slightly hygroscopic and has a water content of approximately 5.5 percent by weight, which indicates that form C is a monohydrate. The hydrate C has a melting point near 94° C. ( $\Delta H_f$  is about 31 J/g) and hydrate form C is especially suitable as intermediate and starting material to produce stable polymorphic forms. Polymorph form C can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Still another object of the invention is a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

8.6 (s), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.17 (m), 3.05 (s), 2.88 (m), and 2.79 (m),

hereinafter designated as form a

In a more preferred embodiment, the present invention comprises a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

8.6 (s), 6.8 (w), 5.56 (m), 4.99 (m), 4.67 (s), 4.32 (m), 3.93 (vs), 3.88 (w), 3.64 (w), 3.41 (w), 3.25 (w), 3.17 (m), 3.05 (s), 2.94 (w), 2.92 (w), 2.88 (m), 2.85 (w), 2.80 (w), 2.79 (m), 2.68 (w), 2.65 (w), 2.52 (vw), 2.35 (w), 2.34 (w), 2.30 (w), and 2.29 (w),

hereinafter designated as form D.

In still another preferred embodiment, the present invention comprises a crystalline hydrate D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 4.

The hydrate form D is slightly hygroscopic and may have a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form D is a monohydrate. The hydrate D has a melting point near 153° C. ( $\Delta H_f$  is about 111 J/g) and is of much higher stability than form C and is even stable when exposed to air humidity at ambient temperature. Hydrate form D can therefore either be used to prepare formulations or as intermediate and starting material to produce stable polymorph forms. Polymorph form D can be prepared as a solid

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powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Still another object of the invention is a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.4 (s), 4.87 (w), 3.69 (m), 3.33 (s), 3.26 (vs), 3.08 (m), 2.95 (m), and 2.87 (m),

hereinafter designated as form E.

In a more preferred embodiment, the present invention comprises a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.4 (s), 6.6 (w), 6.5 (w), 5.95 (vw), 5.61 (vw), 5.48 (w), 5.24 (w), 4.87 (w), 4.50 (vw), 4.27 (w), 3.94 (w), 3.78 (w), 3.69 (m), 3.60 (w), 3.33 (s), 3.26 (vs), 3.16 (w), 3.08 (m), 2.98 (w), 2.95 (m), 2.91 (w), 2.87 (m), 2.79 (w), 2.74 (w), 2.69 (w), and 2.62 (w),

hereinafter designated as form E.

In still another preferred embodiment, the present invention comprises a crystalline hydrate E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 5.

The hydrate form E has a water content of approximately 10 to 14 percent by weight, which suggests that form E is a dihydrate. The hydrate E is formed at temperatures below room temperature. Hydrate form E is especially suitable as intermediate and starting material to produce stable polymorph forms. It is especially suitable to produce the waterfree form J upon drying under nitrogen or optionally under vacuum. Form E is non-hygroscopic and stable under rather high relative humidities, i.e., at relative humidities above about 60% and up to about 85%. Polymorph form E can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Still another object of the invention is a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.8 (vs), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 2.96 (m), 2.89 (m), and 2.67 (m),

hereinafter designated as form H.

In a more preferred embodiment, the present invention comprises a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.8 (vs), 10.3 (w), 8.0 (w), 6.6 (w), 6.07 (w), 4.81 (w), 4.30 (w), 3.87 (m), 3.60 (m), 3.27 (m), 3.21 (m), 3.13 (w), 3.05 (w), 2.96 (m), 2.89 (m), 2.82 (w), and 2.67 (m),

hereinafter designated as form H.

In still another preferred embodiment, the present invention comprises a crystalline hydrate H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 8.

The hydrate form H has a water content of approximately 5.0 to 7.0 percent by weight, which suggests that form H is a hygroscopic monohydrate. The hydrate form H is formed at temperatures below room temperature. Hydrate form H is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form H can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.



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Still another object of the invention is a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

8.8 (m), 6.3 (m), 5.65 (m), 5.06 (m), 4.00 (m), 3.88 (m), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), and 3.04 (vs),

hereinafter designated as form O.

In a more preferred embodiment, the present invention comprises a crystalline hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

15.9 (w), 14.0 (w), 12.0 (w), 8.8 (m), 7.0 (w), 6.5 (w), 6.3 (m), 6.00 (w), 5.75 (w), 5.65 (m), 5.06 (m), 4.98 (m), 4.92 (m), 4.84 (w), 4.77 (w), 4.42 (w), 4.33 (w), 4.00 (m), 3.88 (m), 3.78 (w), 3.69 (s), 3.64 (s), 3.52 (vs), 3.49 (s), 3.46 (s), 3.42 (s), 3.32 (m), 3.27 (m), 3.23 (s), 3.18 (s), 3.15 (vs), 3.12 (m), 3.04 (vs), 2.95 (m), 2.81 (s), 2.72 (m), 2.67 (m), and 2.61 (m),

hereinafter designated as form O.

In still another preferred embodiment, the present invention comprises a crystalline hydrate O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 15.

The hydrate form O is formed at temperatures near room temperature. Hydrate form O is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form O can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

## 2. Solvate Forms of

### (6R)-L-erythro-tetrahydrobiopterin dihydrochloride

(6R)-L-erythro-tetrahydrobiopterin dihydrochloride forms crystalline solvate forms G, I, L, M and N, depending from the solvent used in the preparation method.

Still another object of the invention is a crystalline ethanol solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.5 (vs), 7.0 (w), 4.41 (w), 3.63 (m), 3.57 (m), 3.49 (w), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w),

hereinafter designated as form G.

In a more preferred embodiment, the present invention comprises a crystalline ethanol solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.5 (vs), 10.9 (w), 9.8 (w), 7.0 (w), 6.3 (w), 5.74 (w), 5.24 (vw), 5.04 (vw), 4.79 (w), 4.41 (w), 4.02 (w), 3.86 (w), 3.77 (w), 3.69 (w), 3.63 (m), 3.57 (m), 3.49 (m), 3.41 (m), 3.26 (m), 3.17 (m), 3.07 (m), 2.97 (m), 2.95 (m), 2.87 (w), and 2.61 (w),

hereinafter designated as form G.

In still another preferred embodiment, the present invention comprises a crystalline solvate G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 7.

The ethanol solvate form G has an ethanol content of approximately 8.0 to 12.5 percent by weight, which suggests that form G is a hygroscopic mono ethanol solvate. The solvate form G is formed at temperatures below room tem-

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perature. Form G is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form G can be prepared as a solid powder with a desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Still another object of the invention is a crystalline acetic acid solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.5 (m), 3.67 (vs), 3.61 (m), 3.44 (m), 3.11 (s), and 3.00 (m),

hereinafter designated as form I.

In a more preferred embodiment, the present invention comprises a crystalline acetic acid solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.5 (m), 14.0 (w), 11.0 (w), 7.0 (vw), 6.9 (vw), 6.2 (vw), 5.30 (w), 4.79 (w), 4.44 (w), 4.29 (w), 4.20 (vw), 4.02 (w), 3.84 (w), 3.80 (w), 3.67 (vs), 3.61 (m), 3.56 (w), 3.44 (m), 3.27 (w), 3.19 (w), 3.11 (s), 3.00 (m), 2.94 (w), 2.87 (w), and 2.80 (w),

hereinafter designated as form I.

In still another preferred embodiment, the present invention comprises a crystalline acetic acid solvate I of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 9.

The acetic acid solvate form I has an acetic acid content of approximately 12.7 percent by weight, which suggests that form I is a hygroscopic acetic acid mono solvate. The solvate form I is formed at temperatures below room temperature. Acetic acid solvate form I is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form I can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu$ m to about 500  $\mu$ m.

Still another object of the invention is a crystalline mixed ethanol solvate/hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.1 (vs), 10.4 (w), 6.9 (w), 6.5 (w), 6.1 (w), 4.71 (w), 3.46 (m), 3.36 (m), and 2.82 (w),

hereinafter designated as form L.

In a more preferred embodiment, the present invention comprises a crystalline mixed ethanol solvate/hydrate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values (Å):

14.1 (vs), 10.4 (w), 9.5 (w), 9.0 (vw), 6.9 (w), 6.5 (w), 6.1 (w), 5.75 (w), 5.61 (w), 5.08 (w), 4.71 (w), 3.86 (w), 3.78 (w), 3.46 (m), 3.36 (m), 3.06 (w), 2.90 (w), and 2.82 (w),

hereinafter designated as form L.

In still another preferred embodiment, the present invention comprises a crystalline mixed ethanol solvate/hydrate L of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 12.

Form L may contain 4% but up to 13% ethanol and 0% to about 6% of water. Form L may be transformed into form G when treated in ethanol at temperatures from about 0° C. to 20° C. In addition form L may be transformed into form B when treated in an organic solvent at ambient temperatures (10° C. to 60° C.). Polymorph form L can be prepared as a

solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Still another object of the invention is a crystalline ethanol solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

18.9 (s), 6.4 (m), and 3.22 (vs),

hereinafter designated as form M.

In a more preferred embodiment, the present invention comprises a crystalline ethanol solvate of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

18.9 (s), 6.4 (m), 6.06 (w), 5.66 (w), 5.28 (w), 4.50 (w), 4.23 (w), and 3.22 (vs),

hereinafter designated as form M.

In still another preferred embodiment, the present invention comprises a crystalline ethanol solvate M of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 13.

Form M may contain 4% but up to 13% ethanol and 0% to about 6% of water, which suggests that form M is a slightly hygroscopic ethanol solvate. The solvate form M is formed at room temperature. Form M is especially suitable as intermediate and starting material to produce stable polymorph forms, since form M can be transformed into form G when treated in ethanol at temperatures between about  $-10^{\circ}\text{C}$ ., and into form B when treated in organic solvents such as ethanol, C3 and C4 alcohols, or cyclic ethers such as THF and dioxane. Polymorph form M can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

Still another object of the invention is a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

19.5 (m), 6.7 (w), 3.56 (m), and 3.33 (vs), 3.15 (w),

hereinafter designated as form N.

In a more preferred embodiment, the present invention comprises a crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern with characteristic peaks expressed in d-values ( $\text{\AA}$ ):

19.5 (m), 9.9 (w), 6.7 (w), 5.15 (w), 4.83 (w), 3.91 (w), 3.56 (m), 3.33 (vs), 3.15 (w), 2.89 (w), 2.81 (w), 2.56 (w), and 2.36 (w),

hereinafter designated as form N.

In still another preferred embodiment, the present invention comprises a crystalline polymorph N of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, which exhibits a characteristic X-ray powder diffraction pattern as exhibited in FIG. 14.

Form N may contain in total up to 10% of isopropanol and water, which suggests that form N is a slightly hygroscopic isopropanol solvate. Form N may be obtained through washing of form D with isopropanol and subsequent drying in vacuum at about  $30^{\circ}\text{C}$ . Form N is especially suitable as intermediate and starting material to produce stable polymorph forms. Polymorph form N can be prepared as a solid powder with desired medium particle size range which is typically ranging from 1  $\mu\text{m}$  to about 500  $\mu\text{m}$ .

For the preparation of the polymorph forms, there may be used crystallisation techniques well known in the art, such as stirring of a suspension (phase equilibration in), precipitation, re-crystallisation, evaporation, solvent like water sorption methods or decomposition of solvates. Diluted, saturated or

super-saturated solutions may be used for crystallisation, with or without seeding with suitable nucleating agents. Temperatures up to  $100^{\circ}\text{C}$ . may be applied to form solutions. Cooling to initiate crystallisation and precipitation down to  $-100^{\circ}\text{C}$ . and preferably down to  $-30^{\circ}\text{C}$ . may be applied. Meta-stable polymorphs or pseudo-polymorphic forms can be used to prepare solutions or suspensions for the preparation of more stable forms and to achieve higher concentrations in the solutions.

#### 4. Preparation of Polymorph Forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride

##### Polymorph Form A

Polymorph form A may be obtained by freeze drying or water removal of solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water. A further object of the invention is a process for the preparation of polymorph form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at ambient temperatures in water, (1) cooling the solution to low temperatures for solidifying the solution, and removing water under reduced pressure, or (2) removing water from said aqueous solution.

The crystalline form A can be isolated by filtration and then dried to evaporate absorbed water from the product. Drying conditions and methods are known and drying of the isolated product or water removal pursuant to variant (2) according to the invention may be carried out in applying elevated temperatures, for example up to  $80^{\circ}\text{C}$ ., preferably in the range from  $30^{\circ}\text{C}$ . to  $80^{\circ}\text{C}$ ., under vacuum or elevated temperatures and vacuum. Prior to isolation of a precipitate obtained in variant (2), the suspension may be stirred for a certain time for phase equilibration. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 5 to 40 percent by weight, referred to the solution.

Ambient temperatures may mean a range from 30 to  $120^{\circ}\text{C}$ . Low temperatures may mean temperatures below  $-40^{\circ}\text{C}$ . and preferably below  $-60^{\circ}\text{C}$ . and to  $-180^{\circ}\text{C}$ . A fast cooling is preferred to obtain solid solutions as starting material. A reduced pressure is applied until the solvent is completely removed. Freeze drying is a technology well known in the art. The time to complete solvent removal is dependent on the applied vacuum, which may be from 0.01 to 1 mbar, the solvent used and the freezing temperature.

Polymorph form A is stable at room temperature or below room temperature under substantially water free conditions, which is demonstrated with phase equilibration tests of suspensions in tetrahydrofuran or tertiary-butyl methyl ether stirred for five days and 18 hours respectively under nitrogen at room temperature. Filtration and air drying at room temperature yields unchanged polymorph form A.

##### Polymorph B

All crystal forms (polymorphs, hydrates and solvates), inclusive crystal form B, can be used for the preparation of the most stable polymorph B.

Polymorph B may be obtained by phase equilibration of suspensions of amorphous or other forms than polymorph form B, such as polymorph A, in suitable polar and non aqueous solvents. The present invention also refers to a process for the preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dispersion of particles of a solid form, preferably other than form B, of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent at room temperature, stirring the suspension at ambient temperatures for a time sufficient to produce poly-



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morph form B, thereafter isolating crystalline form B and removing the solvent from the isolated form B.

Ambient temperatures may mean temperatures in a range from 0° C. to 60° C., preferably 20° C. to 40° C. The applied temperature may be changed during treatment and stirring by decreasing the temperature stepwise or continuously. Suitable solvents are for example methanol, ethanol, isopropanol, other C<sub>3</sub>- and C<sub>4</sub>-alcohols, acetic acid, acetonitrile, tetrahydrofuran, methyl-t-butyl ether, 1,4-dioxane, ethyl acetate, isopropyl acetate, other C<sub>3</sub>-C<sub>6</sub>-acetates, methyl ethyl ketone and other methyl-C<sub>3</sub>-C<sub>5</sub>-alkyl-ketones. The time to complete phase equilibration may be up to 30 hours and preferably up to 20 hours, or less than 20 hours.

Polymorph B may also be obtained by crystallisation from solvent mixtures containing up to about 5% water, especially from mixtures of ethanol, acetic acid and water. The present invention also refers to a process for the preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolution, optionally at elevated temperatures, preferably of a solid lower energy form than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a solvent mixture comprising ethanol, acetic acid and water, addition of seeds to the solution, cooling the obtained suspension and isolation of the formed crystals.

Dissolution may be carried out at room temperature or up to 70° C., preferably up to 50° C. There may be used the final solvent mixture for dissolution or the starting material may be first dissolved in water and the other solvents may than be added both or one after the other solvent. The composition of the solvent mixture may comprise a volume ratio of water: acetic acid:tetrahydrofuran of 1:3:2 to 1:9:4 and preferably 1:5:4. The solution is preferably stirred. Cooling may mean temperatures down to -40° C. to 0° C., preferably down to 10° C. to 30° C. Suitable seeds are polymorph form B from another batch or crystals having a similar or identical morphology. After isolation, the crystalline form B can be washed with a non-solvent such as acetone or tetrahydrofuran and dried in usual manner.

Polymorph B may also be obtained by crystallisation from aqueous solutions through the addition of non-solvents such as methanol, ethanol and acetic acid. The crystallisation and isolation procedure can be advantageously carried out at room temperature without cooling the solution. This process is therefore very suitable to be carried out at an industrial scale.

In a preferred embodiment, the present invention refers to a process for the preparation of polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolution of a solid form other than form B or of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water at ambient temperatures, adding a non-solvent in an amount sufficient to form a suspension, optionally stirring the suspension for a certain time, and thereafter isolation of the formed crystals.

A crystallization experiment from solution can be followed by a subsequent suspension equilibration under ambient conditions.

Ambient temperatures may mean a temperature in the range of 10 to 40° C., and most preferably room temperature. The concentration of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in the aqueous solution may be from 10 to 80 percent by weight, more preferably from 20 to 60 percent by weight, referred to the solution. Preferred non-solvents are methanol, ethanol and acetic acid. The non-solvent may be added to the aqueous solution. More preferably, the aqueous solution is added to the non-solvent. The stirring time after formation of the suspension may be up to 30 hours and preferably

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erably up to 20 hours or less than 20 hours. Isolation by filtration and drying is carried out in known manner as described before.

Polymorph form B is a very stable crystalline form, that can be easily filtered off, dried and ground to particle sizes desired for pharmaceutical formulations. These outstanding properties renders polymorph form B especially feasible for pharmaceutical application.

## Polymorph F

Polymorph F may be obtained by phase equilibration of suspensions of polymorph form A in suitable polar and non-aqueous solvents, which scarcely dissolve said lower energy forms, especially alcohols such as methanol, ethanol, propanol and isopropanol. The present invention also refers to a process for the preparation of polymorph form F of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dispersion of particles of solid form A of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-aqueous solvent that scarcely dissolves said (6R)-L-erythro-tetrahydrobiopterin dihydrochloride below room temperature, stirring the suspension at said temperatures for a time sufficient to produce polymorph form F, thereafter isolating crystalline form F and removing the solvent from the isolated form F. Removing of solvent and drying may be carried out under air, dry air or a dry protection gas such as nitrogen or noble gases and at or below room temperature, for example down to 0° C. The temperature during phase equilibration is preferably from 5 to 15° C. and most preferably about 10° C.

## Polymorph J

Polymorph J may be obtained by dehydration of form E at moderate temperatures under vacuum. The present invention also refers to a process for the preparation of polymorph form J of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising preparation of form E and removing the water from form E by treating form E in a vacuum drier to obtain form J at moderate temperatures which may mean a temperature in the range of 25 to 70° C., and most preferably 30 to 50° C.

## Polymorph K

Polymorph K may be obtained by crystallization from mixtures of polar solvents containing small amounts of water and in the presence of small amounts of ascorbic acid. Solvents for the solvent mixture may be selected from acetic acid and an alcohol such as methanol, ethanol, n- or isopropanol. The present invention also refers to a process for the preparation of polymorph form K of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and an alcohol or tetrahydrofuran containing small amounts of water and a small amount of ascorbic acid at elevated temperatures, lowering temperature below room temperature to crystallise said dihydrochloride, isolating the precipitate and drying the isolated precipitate at elevated temperature optionally under vacuum. Suitable alcohols are for example methanol, ethanol, propanol and isopropanol, whereby ethanol is preferred. The ratio of acetic acid to alcohol or tetrahydrofuran may be from 2:1 to 1:2 and preferably about 1:1. Dissolution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride can be carried out in presence of a higher water content and more of the antisolvent mixture can be added to obtain complete precipitation. The amount of water in the final composition may be from 0.5 to 5 percent by weight and the amount of ascorbic acid may be from 0.01 to 0.5 percent by weight, both referred to the solvent mixture. The temperature for dissolution may be in the range from 30 to 100 and preferably 35 to 70° C. and the drying temperature may be in the range from 30 to 50° C. The precipitate may be

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washed with an alcohol such as ethanol after isolation, e.g. filtration. The polymorph K can easily be converted in the most stable form B by phase equilibration in e.g. isopropanol and optionally seeding with form B crystals at above room temperature such as temperatures from 30 to 40° C.

#### 5. Preparation of Hydrate Forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride

##### Form C

Hydrate form C may be obtained by phase equilibration at ambient temperatures of a polymorph form such as polymorph B suspension in a non-solvent which contains water in an amount of preferably about 5 percent by weight, referred to the solvent. The present invention also refers to a process for the preparation of hydrate form C of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising suspending (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a non-solvent such as heptane, C<sub>1</sub>-C<sub>4</sub>-alcohols such as methanol, ethanol, 1- or 2-propanol, acetates, such as ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or binary or ternary mixtures of such non-solvents, to which sufficient water is added to form a monohydrate, and stirring the suspension at or below ambient temperatures (e.g. 0 to 30° C.) for a time sufficient to form a monohydrate. Sufficient water may mean from 1 to 10 and preferably from 3 to 8 percent by weight of water, referred to the amount of solvent. The solids may be filtered off and dried in air at about room temperature. The solid can absorb some water and therefore possess a higher water content than the theoretical value of 5.5 percent by weight. Hydrate form C is unstable with respect to forms ID and B, and easily converted to polymorph form B at temperatures of about 40° C. in air and lower relative humidity. Form C can be transformed into the more stable hydrate D by suspension equilibration at room temperature.

##### Form D

Hydrate form D may be obtained by adding at about room temperature concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents, and stirring the suspension at ambient temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. The present invention also refers to a process for the preparation of hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising adding at about room temperature a concentrated aqueous solution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent and stifling the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. Form D contains a small excess of water, related to the monohydrate, and it is believed that it is absorbed water due to the slightly hygroscopic nature of this crystalline hydrate. Hydrate form D is deemed to be the most stable one under the known hydrates at ambient temperatures and a relative humidity of less than 70%. Hydrate form D may be used for formulations prepared under conditions, where this hydrate is stable. Ambient temperature may mean 20 to 30° C.

##### Hydrate form E

Hydrate form E may be obtained by adding concentrated aqueous solutions of (6R)-L-erythro-tetrahydrobiopterin

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dihydrochloride to an excess of a non-solvent cooled to temperatures from about 10 to -10° C. and preferably between 0 to 10° C. and stirring the suspension at said temperatures. The crystalline solid can be filtered off and then dried under dry nitrogen at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is isopropanol. The addition of the aqueous solution may be carried out drop-wise to avoid a sudden precipitation. The present invention also refers to a process for the preparation of hydrate form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising adding a concentrated aqueous solution of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride to an excess of a non-solvent which is cooled to temperatures from about 10 to -10° C., and stifling the suspension at ambient temperatures. Excess of non-solvent may mean a ratio of aqueous to the non solvent from 1:10 to 1:1000. A preferred non-solvent is tetrahydrofuran. Another preparation process comprises exposing polymorph form B to an air atmosphere with a relative humidity of 70 to 90%, preferably about 80%. Hydrate form E is deemed to be a dihydrate, whereby some additional water may be absorbed. Polymorph form E can be transformed into polymorph J upon drying under vacuum at moderate temperatures, which may mean between 20° C. and 50° C. at pressures between 0 and 100 mbar. Form E is especially suitable for formulations in semi solid forms because of its stability at high relative humidities.

##### Form H

Hydrate form H may be obtained by dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water, adding then a non-solvent to precipitate a crystalline solid, cooling the obtained suspension and stirring the cooled suspension for a certain time. The crystalline solid is filtered off and then dried under vacuum at ambient temperatures. Non-solvents are for example such as hexane, heptane, dichloromethane, 1- or 2-propanol, acetone, ethyl acetate, acetonitrile, acetic acid or ethers such as tetrahydrofuran, dioxane, tertiary-butyl methyl ether, or mixtures of such non-solvents. A preferred non-solvent is tetrahydrofuran. The present invention also refers to a process for the preparation of hydrate form H of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolving at ambient temperatures (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and a less amount than that of acetic acid of water, adding a non-solvent and cooling the obtained suspension to temperatures in the range of -10 to 10° C., and preferably -5 to 5° C., and stifling the suspension at said temperature for a certain time. Certain time may mean 1 to 20 hours. The weight ratio of acetic acid to water may be from 2:1 to 25:1 and preferably 5:1 to 15:1. The weight ratio of acetic acid/water to the non-solvent may be from 1:2 to 1:5. Hydrate form H seems to be a monohydrate with a slight excess of water absorbed due to the hygroscopic nature.

##### Form O

Hydrate form O can be prepared by exposure of polymorphic form F to a nitrogen atmosphere containing water vapour with a resulting relative humidity of about 52% for about 24 hours. The fact that form F, which is a slightly hygroscopic anhydrate, can be used to prepare form O under 52% relative humidity suggests that form O is a hydrate, which is more stable than form F under ambient temperature and humidity conditions.

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# 6. Preparation of Solvate Forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride

## Form G

Ethanol solvate form G may be obtained by crystallisation of L-erythro-tetrahydrobiopterin dihydrochloride dissolved in water and adding a large excess of ethanol, stirring the obtained suspension at or below ambient temperatures and drying the isolated solid under air or nitrogen at about room temperature. Here, a large excess of ethanol means a resulting mixture of ethanol and water with less than 10% water, preferably about 3 to 6%. The present invention also refers to a process for the preparation of ethanolate form G of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, comprising dissolving at about room temperature to temperatures of 75° C. (6R)-L-erythro-tetrahydrobiopterin dihydrochloride in water or in a mixture of water and ethanol, cooling a heated solution to room temperature and down to 5 to 10° C., adding optionally ethanol to complete precipitation, stirring the obtained suspension at temperatures of 20 to 5° C., filtering off the white, crystalline solid and drying the solid under air or a protection gas such as nitrogen at temperatures about room temperature. The process may be carried out in a first variant in dissolving (6R)-L-erythro-tetrahydrobiopterin dihydrochloride at about room temperature in a lower amount of water and then adding an excess of ethanol and then stirring the obtained suspension for a time sufficient for phase equilibration. In a second variant, (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be suspended in ethanol, optionally adding a lower amount of water, and heating the suspension and dissolute (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, cooling down the solution to temperatures of about 5 to 15° C., adding additional ethanol to the suspension and then stirring the obtained suspension for a time sufficient for phase equilibration.

## Form I

Acetic acid solvate form I may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in a mixture of acetic acid and water at elevated temperature, adding further acetic acid to the solution, cooling down to a temperature of about 10° C., then warming up the formed suspension to about 15° C., and then stirring the obtained suspension for a time sufficient for phase equilibration, which may last up to 3 days. The crystalline solid is then filtered off and dried under air or a protection gas such as nitrogen at temperatures about room temperature.

## Form L

Form L may be obtained by suspending hydrate form E at room temperature in ethanol and stirring the suspension at temperatures from 0 to 10° C., preferably about 5° C., for a time sufficient for phase equilibration, which may be 10 to 20 hours. The crystalline solid is then filtered off and dried preferably under reduced pressure at 30° C. or under nitrogen. Analysis by TG-FTIR suggests that form L may contain variable amounts of ethanol and water, i.e. it can exist as a polymorph (anhydrite), as a mixed ethanol solvate/hydrate, or even as a hydrate.

## Form M

Ethanol solvate form M may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in ethanol and evaporation of the solution under nitrogen at ambient temperature, i.e., between 10° C. and 40° C. Form M may also be obtained by drying of form G under a slight flow of dry nitrogen at a rate of about 20 to 100 ml/min. Depending on the extent of drying under nitrogen, the remaining amount of ethanol may be variable, i.e. from about 3% to 13%.

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## Form N

The isopropanol form N may be obtained by dissolution of L-erythro-tetrahydrobiopterin dihydrochloride in 4.0 ml of a mixture of isopropanol and water (mixing volume ratio for example 4:1). To this solution is slowly added isopropanol (IPA, for example about 4.0 ml) and the resulting suspension is cooled to 0° C. and stirred for several hours (e.g. about 10 to 18 hours) at this temperature. The suspension is filtered and the solid residue washed with isopropanol at room temperature. The obtained crystalline material is then dried at ambient temperature (e.g. about 20 to 30° C.) and reduced pressure (about 2 to 10 mbar) for several hours (e.g. about 5 to 20 hours). TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water. This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as a non-solvated form containing a small amount of water.

A further object of the invention is a pharmaceutical composition comprising solid crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride selected from the group consisting of forms A, B, D, E, F, J, K, L and O or a combination thereof, and a pharmaceutically acceptable carrier or diluent.

As mentioned above, it was found that crystal form B is the most stable form of all found crystal forms. Crystal form B is especially suitable for various types and a broad range of formulations, even in presence of humid components without formation of hydrates.

Accordingly, this invention is also directed to a pharmaceutical composition comprising a pure polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

In principle, also forms A, D, E, F, J, K, L and O are suitable for use in pharmaceutical formulations and accordingly, this invention is also directed to a pharmaceutical composition comprising forms A, D, E, F, J, K, L and O of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent. For forms A, F, J, K and L are preferably used dry formulation components and products may be kept in sealed containers, mainly to avoid formation of hydrates. Hydrate forms D, E and O can be used directly in presence of humid components for the formulation and air humidity must not be excluded.

It was surprisingly found that hydrate form D is the most stable form under the hydrates and forms B and D are especially suitable to be used in pharmaceutical formulations. Forms B and D presents some advantages like an aimed manufacture, good handling due to convenient crystal size and morphology, very good stability under production conditions of various types of formulation, storage stability, higher solubility, and high bio-availability.

Accordingly, this invention is particularly directed to a pharmaceutical composition comprising polymorph form B or hydrate form D of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride and a pharmaceutically acceptable carrier or diluent.

In the following, crystal form is meaning A, B, D, E, F, J, K, L and O.

The amount of crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride substantially depends on type of formulation and desired dosages during administration time periods. The amount in an oral formulation may be from 0.1 to 50 mg, preferably from 0.5 to 30 mg, and more preferably from 1 to 15 mg.

The crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride may be used together with folates such as,



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follic acid, or tetrahydrofolates. Examples of tetrahydrofolates are tetrahydrofollic acid, 5,10-methylenetetrahydrofollic acid, 10-formyltetrahydrofollic acid, 5-formyltetrahydrofollic acid or preferably 5-methyltetrahydrofollic acid, their polyglutamates, their optically pure diastereoisomers, but also mixtures of diastereoisomers, especially the racemic mixture, pharmaceutically acceptable salts such as sodium, potassium, calcium or ammonium salts, each alone, in combination with an other folate or additionally with arginine. The weight ratio of crystal forms:follic acids or salts thereof: arginine may be from 1:10:10 to 10:1:1.

Oral formulations may be solid formulations such as capsules, tablets, pills and troches, or liquid formulations such as aqueous suspensions, elixirs and syrups. Solid and liquid formulations encompass also incorporation of crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride according to the invention into liquid or solid food. Liquids also encompass solutions of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride for parenteral applications such as infusion or injection.

The crystal form according to the invention may be directly used as powder (micronized particles), granules, suspensions or solutions, or it may be combined together with other pharmaceutically acceptable ingredients in admixing the components and optionally finely divide them, and then filling capsules, composed for example from hard or soft gelatine, compressing tablets, pills or troches, or suspend or dissolve them in carriers for suspensions, elixirs and syrups. Coatings may be applied after compression to form pills.

Pharmaceutically acceptable ingredients are well known for the various types of formulation and may be for example binders such as natural or synthetic polymers, excipients, lubricants, surfactants, sweetening and flavouring agents, coating materials, preservatives, dyes, thickeners, adjuvants, antimicrobial agents, antioxidants and carriers for the various formulation types.

Examples for binders are gum tragacanth, acacia, starch, gelatine, and biological degradable polymers such as homo- or co-polyesters of dicarboxylic acids, alkylene glycols, polyalkylene glycols and/or aliphatic hydroxyl carboxylic acids; homo- or co-polyamides of dicarboxylic acids, alkylene diamines, and/or aliphatic amino carboxylic acids; corresponding polyester-polyamide-co-polymers, polyanhydrides, polyorthoesters, polyphosphazene and polycarbonates. The biological degradable polymers may be linear, branched or crosslinked. Specific examples are poly-glycolic acid, poly-lactic acid, and poly-D,L-lactide/glycolide. Other examples for polymers are water-soluble polymers such as polyoxaalkylenes (polyoxaethylene, polyoxapropylene and mixed polymers thereof, poly-acrylamides and hydroxylalkylated polyacrylamides, poly-maleic acid and esters or -amides thereof, poly-acrylic acid and esters or -amides thereof, poly-vinylalcohol und esters or -ethers thereof, poly-vinylimidazole, poly-vinylpyrrolidon, und natural polymers like chitosan.

Examples for excipients are phosphates such as dicalcium phosphate.

Examples for lubricants are natural or synthetic oils, fats, waxes, or fatty acid salts like magnesium stearate.

Surfactants may be anionic, anionic, amphoteric or neutral. Examples for surfactants are lecithin, phospholipids, octyl sulfate, decyl sulfate, dodecyl sulfate, tetradecyl sulfate, hexadecyl sulfate and octadecyl sulfate, Na oleate or Na caprate, 1-acylaminoethane-2-sulfonic acids, such as 1-octanoylaminoethane-2-sulfonic acid, 1-decanoylaminoethane-2-sulfonic acid, 1-dodecanoylaminoethane-2-sulfonic acid, 1-tetradecanoylaminoethane-2-sulfonic acid, 1-hexade-

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canoylaminoethane-2-sulfonic acid, and 1-octadecanoylaminoethane-2-sulfonic acid, and taurocholic acid and taurodeoxycholic acid, bile acids and their salts, such as cholic acid, deoxycholic acid and sodium glycocholates, sodium caprate or sodium laurate, sodium oleate, sodium lauryl sulphate, sodium cetyl sulphate, sulfated castor oil and sodium diocetylsulfosuccinate, cocamidopropylbetaine and laurylbetaine, fatty alcohols, cholesterol, glycerol mono- or -distearate, glycerol mono- or -dioleate and glycerol mono- or -dipalmitate, and polyoxyethylene stearate.

Examples for sweetening agents are sucrose, fructose, lactose or aspartam.

Examples for flavouring agents are peppermint, oil of wintergreen or fruit flavours like cherry or orange flavour.

Examples for coating materials are gelatine, wax, shellac, sugar or biological degradable polymers.

Examples for preservatives are methyl or propylparabens, sorbic acid, chlorobutanol, phenol and thimerosal.

Examples for adjuvants are fragrances.

Examples for thickeners are synthetic polymers, fatty acids and fatty acid salts and esters and fatty alcohols.

Examples for antioxidants are vitamins, such as vitamin A, vitamin C, vitamin D or vitamin E, vegetable extracts or fish oils.

Examples for liquid carriers are water, alcohols such as ethanol, glycerol, propylene glycol, liquid polyethylene glycols, triacetin and oils. Examples for solid carriers are talc, clay, microcrystalline cellulose, silica, alumina and the like.

The formulation according to the invention may also contain isotonic agents, such as sugars, buffers or sodium chloride.

The hydrate form D according to the invention may also be formulated as effervescent tablet or powder, which disintegrate in an aqueous environment to provide a drinking solution.

A syrup or elixir may contain the polymorph of the invention, sucrose or fructose as sweetening agent a preservative like methylparaben, a dye and a flavouring agent.

Slow release formulations may also be prepared from the polymorph according to the invention in order to achieve a controlled release of the active agent in contact with the body fluids in the gastro intestinal tract, and to provide a substantial constant and effective level of the active agent in the blood plasma. The crystal form may be embedded for this purpose in a polymer matrix of a biological degradable polymer, a water-soluble polymer or a mixture of both, and optionally suitable surfactants. Embedding can mean in this context the incorporation of micro-particles in a matrix of polymers. Controlled release formulations are also obtained through encapsulation of dispersed micro-particles or emulsified micro-droplets via known dispersion or emulsion coating technologies.

The crystal form of this invention is also useful for administering a combination of therapeutic effective agents to an animal. Such a combination therapy can be carried out in using at least one further therapeutic agent which can be additionally dispersed or dissolved in a formulation.

The crystal form of this invention and its formulations respectively can be also administered in combination with other therapeutic agents that are effective to treat a given condition to provide a combination therapy.

The crystal form and the pharmaceutical composition according to the invention are highly suitable for effective treatment of neurological disorders.

Another object of the invention is a method of delivering crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydro-

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chloride according to the invention to a host, comprising administering to a host an effective amount of a polymorph according to the invention.

A further object of the invention is the use of crystal forms of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride for the manufacture of a medicament useful in the treatment of neurological disorders.

The following examples illustrate the invention without limiting the scope.

## A) Preparation of Polymorph Forms

Within the Examples A1, A5, A6 and A7 (6R)-L-erythro-tetrahydrobiopterin dihydrochloride from Schircks Laboratories, CH-8645 Jona, Switzerland was used as starting material.

## EXAMPLE A1

Preparation of Polymorph Form A of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

1.05 gram of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride are dissolved in 4.0 ml of bi-distilled water at  $23 \pm 2^\circ$  C. The solution is filtrated through a  $0.22 \mu\text{m}$  millipore filtration unit and the filtrate is transferred into a 250 ml round flask. The solution in this flask is frozen by placing the flask into a bed with solid carbon dioxide at  $-78^\circ$  C. The flask with the frozen content is then connected to a laboratory freeze dryer operating at a starting pressure of about 0.05 mbar. After about 20 hours the freeze drying is complete and the vacuum flask is disconnected from the freeze dryer and about 1.0 g of white, crystalline solid material is obtained. Investigation of the obtained solid by powder X-ray diffraction reveals form A, which shows the powder X-ray diffraction pattern as exhibited in table 1 and FIG. 1. Further investigation of the obtained solid by thermogravimetry coupled with infrared spectroscopy at a heating rate of  $10^\circ$  C./minute reveals a water content of about 3% with a nearly continuous release of the water between  $50^\circ$  C. and  $200^\circ$  C. The sample begins to decompose above  $200^\circ$  C.

TABLE 1

D-Spacing for form A		
Angle [ $^\circ 2\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
5.7	15.5	vs
7.4	12.0	m
13.3	6.7	m
13.6	6.5	m
14.0	6.3	w
14.4	6.1	w
14.9	5.96	w
16.1	5.49	m
18.1	4.89	m
23.5	3.79	m
24.0	3.70	s
25.6	3.48	m
25.8	3.45	m
26.8	3.33	s
27.3	3.26	s
27.7	3.22	m
28.1	3.18	m
28.9	3.08	m
29.6	3.02	w
30.3	2.95	w
31.1	2.87	m
32.1	2.79	w
33.2	2.70	w

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## EXAMPLE A2

## Stability of Polymorph Form A

105 mg of polymorph A according to example A1 are suspended in 1.0 ml tertiary butyl methyl ether (TBME). The suspension is stirred under nitrogen atmosphere for about 18 hours at room temperature, filtrated and the white solid residue is then dried under air. Yield: 103 mg of crystalline white solid, which essentially still corresponds to form A according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A3

## Stability of Polymorph Form A

90 mg of polymorph A according to example A1 are suspended in 2.0 ml tetrahydrofuran (THF) and the resulting suspension is stirred in air for five days at room temperature, filtrated and the white solid residue is then dried under air. Yield: 85 mg of crystalline white solid, which still corresponds to form A according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A4

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A

94 mg of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride as polymorph form A according to example A1 are suspended in 1.0 ml of ethanol in a 4.0 ml glass vial under nitrogen. The obtained suspension is stirred at a temperature of  $23^\circ$  C. for about 18 hours. After that time the white suspension is filtrated and the obtained crystalline solid is dried at  $23^\circ$  C. under nitrogen atmosphere for about 1 hour. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form B, which shows the powder X-ray diffraction pattern as exhibited in table 2 and in FIG. 2.

TABLE 2

D-Spacing for form B		
Angle [ $^\circ 2\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
10.1	8.7	vs
12.9	6.9	w
15.0	5.90	vw
15.7	5.63	m
17.5	5.07	m
18.6	4.76	m
20.1	4.40	m
21.4	4.15	w
22.2	4.00	s
22.5	3.95	m
25.3	3.52	m
25.8	3.44	w
26.8	3.32	m
27.6	3.23	s
28.1	3.17	w
28.7	3.11	vs
29.2	3.06	w
29.9	2.99	w
30.1	2.96	w
30.4	2.94	m
31.2	2.87	w
31.5	2.84	s

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TABLE 2-continued

D-Spacing for form B		
Angle [ $^{\circ}2\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
31.7	2.82	m
33.3	2.69	w
34.7	2.59	w
36.9	2.44	w

## EXAMPLE A5

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

337 mg of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride are dissolved in 0.5 ml of bi-distilled water. 300  $\mu\text{l}$  of this aqueous solution are added drop wise into a 22 ml glass vial containing 10.0 ml of ethanol. Upon addition of the aqueous solution to the ethanol, a white suspension is formed that is further stirred at 23 $^{\circ}$  C. for about 15 hours. Thereafter a white, crystalline material is obtained by filtration and drying under nitrogen at 23 $^{\circ}$  C. for about 1 hour. Yield is 74 mg. Investigation of the obtained solid reveals a powder X-ray diffraction pattern and Raman spectrum, which are identical to those described in example A4.

## EXAMPLE A6

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

337 mg of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride are dissolved in 0.5 ml of bi distilled water. 300  $\mu\text{l}$  of this aqueous solution are added drop-wise into a 22 ml glass vial containing 10.0 ml of acetic acid. Upon addition of the aqueous solution to the acetic acid, a white suspension is formed that is further stirred at 23 $^{\circ}$  C. for about 15 hours. Thereafter a white crystalline material is obtained by filtration and drying under nitrogen for about 2 hours and 23 $^{\circ}$  C. Yield is 118 mg. Investigation of the obtained solid by Raman spectroscopy reveals an identical spectrum as described in example A4.

## EXAMPLE A7

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

1.0 g of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride are added to 4 ml bi-distilled water in a test-tube. This aqueous solution is added to 20 ml 100% acetic acid in a glass vial at room temperature. A gelatine-like precipitate is formed that dissolves within several minutes. Then 16 ml tetrahydrofuran are added and the solution is seeded with polymorph B crystals. A suspension is formed during stirring for 10 minutes at room temperature. This suspension is cooled to 0 $^{\circ}$  C. and stands then for 1 hour at this temperature. The precipitate is filtered off, washed with tetrahydrofuran and then dried under vacuum for 17 hours at 20 $^{\circ}$  C. and 10 mbar. There are obtained 0.74 g of beige crystals in the polymorph form B, that reveals a powder X-ray diffraction pattern and Raman spectrum, which are identical to those described in example A4.

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## EXAMPLE A8

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from a Mixture of Hydrate Form C and ethanol  
Solvate Form G

60.5 mg hydrate form C according to example B1 and 60.6 mg ethanol solvate form G according to example C1 are suspended in 1.0 ml ethanol (EtOH) under nitrogen. The slurry is stirred over night at room temperature, filtrated and dried in air. Yield: 96.4 mg white crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A9

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from a Mixture of Polymorph Form B and ethanol  
Solvate Form G

60.4 mg ethanol solvate form G according to example C1 and 60.3 mg polymorph form B according to example A4 are suspended under nitrogen atmosphere in 1.0 ml ethanol, stirred over night at room temperature, filtrated and then dried in air. Yield: 86.4 mg white crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A10

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from a Mixture of Hydrate Form C and Polymorph  
Form B

60.7 mg polymorph form B according to example A4 and 60.5 mg hydrate form C according to example B1 are suspended under nitrogen in 1.0 ml EtOH. The resulting suspension is stirred over night at room temperature, filtrated and dried in air. Yield: 86.6 mg white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A11

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A According to Example A1

105 mg of polymorph form A according to example A1 are suspended in 2.0 ml THF containing 2.5% by weight of water. The suspension is stirred at room temperature under nitrogen atmosphere for about 48 hours, filtrated and dried under nitrogen for 20 hours at room temperature. Yield: 91 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A12

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Hydrate Form E According to Example B8

115 mg of hydrate form E according to example B8 are suspended in 1.5 ml EtOH. The suspension is stirred at room



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temperature under nitrogen atmosphere for about 22 hours, filtrated and dried under nitrogen. Yield: 75 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A13

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

205 mg of polymorph form B according to example A4 are suspended in 2.0 ml isopropanol (IPA) containing 5% by weight of water. The suspension is stirred for 24 hours at room temperature, and then filtered and dried under 53% relative humidity in air. Yield: 116 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A14

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

205 mg of polymorph form B according to example A4 are suspended in 2.0 ml IPA containing 5% by weight of water. The suspension is stirred for 24 hours at 3° C., then filtered and dried under 53% relative humidity in air. Yield: 145 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A15

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A According to Example A1

203 mg polymorph form A according to example A1 are suspended in 2.0 ml IPA and the suspension is stirred at 40° C. for 18 hours, filtered and then dried in air at room temperature. Yield: 192 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A16

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

200 mg polymorph form B according to example A4 are dissolved in 800 µl water. 4.0 ml acetic acid and then 3.0 ml THF added and the resulting suspension is stirred at room temperature for 19 hours. The solid is filtered off and dried in air at room temperature. Yield: 133 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A17

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

256 mg polymorph form B according to example A4 are dissolved in 4.0 ml acetic acid/H<sub>2</sub>O (4:1) and 4.0 ml acetic

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acid are added then. The formed suspension is stirred at 20° C. for about 20 hours, filtered and then dried in air for 4 hours. Yield: 173 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A18

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from acetic acid Solvate Form I According to  
Example C7

51 mg of acetic acid solvate form I according to example C7 is suspended in 1.0 ml EtOH and seeded with 7 mg of form B. The suspension is stirred for 20 hours at room temperature, filtered and dried in air at room temperature. Yield: 52 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A19

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

304 mg of polymorph form B according to example A4 are suspended in 10.0 ml acetic acid and 100 µl water are added. The suspension is cooled to 13° C., seeded with 5 mg form B, stirred at 13° C. for 16 hours, filtered and then dried under nitrogen at room temperature. Yield: 276 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A20

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

304 mg of polymorph form B according to example A4 are suspended in 5.0 ml IPA and 100 µl water are added. The suspension is cooled to 3° C., stirred at 3° C. for 16 hours, filtered and dried in air at room temperature. Yield: 272 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A21

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

296 mg polymorph form B according to example A4 are dissolved in 15 ml methanol at 50° C. The solution is cooled to 5° C. and about 9 ml solvent are evaporated. Stirring of the obtained suspension is then continued at 10° C. for 30 minutes. The suspension is filtered and the solid residue is then dried under nitrogen at room temperature. Yield: 122 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A22

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form K According to Example A28

116 mg of polymorph form K according to example A28 and 7 mg of polymorph form B are suspended in 2.0 ml IPA.

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The suspension is stirred at 35° C. for about 20 hours, filtered and then dried in air at 40° C. for about 1 hour. Yield: 98 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A23

Preparation of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Hydrate Form E According to Example B8

120 mg hydrate form E according to example B8 are suspended in 10 ml EtOH. The obtained suspension is stirred at room temperature for 15 hours, filtered and then dried under nitrogen at room temperature. Yield: 98 mg of white, crystalline solid, which corresponds to form B according to FT Raman spectrum and X-ray diffraction pattern.

## EXAMPLE A24

Stability Test of Polymorph Form B of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

## a) Storage Stability

Polymorph form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is stored during 8 months in a minigrip bag at 40° C. and 75% relative humidity. Purity of the product is determined in different intervals by HPLC. The result is given in table 3.

TABLE 3

	Starting material	After 1 week	After 1 month	After 3 months	After 8 months
HPLC (5 area)	98.4	99.4	98.3	99.1	98.1

The result demonstrates the unusual and unexpected high storage stability of polymorph form B, which makes it especially suitable for preparation of a stable active substance and processing in the manufacture of formulations and storage stable medicaments.

b) Treatment of Polymorph Form B Under the Following Various Conditions Does not Effect the Polymorph Form B, which is Recovered After the Test:

128.2 mg polymorph form B are suspended under nitrogen in 1.0 ml methanol (MeOH). The white suspension is stirred for 5 hours at room temperature, filtrated and dried under nitrogen at room temperature. Yield: 123.4 mg white crystalline solid, polymorph form B.

123.2 mg polymorph form B are suspended under nitrogen in 2.0 ml EtOH. The white suspension is stirred over night at room temperature, filtrated and then dried under nitrogen at room temperature. Yield: 118.6 mg white crystalline solid, polymorph form B.

117.5 mg polymorph form B are suspended under nitrogen in 2.0 ml acetone. The white suspension is stirred over night at room temperature, filtrated and dried under nitrogen room temperature. Yield: 100.3 mg white crystalline solid, polymorph form B.

124.4 mg polymorph form B are suspended under nitrogen in 2.0 ml 2-Propanol. The white suspension is stirred over night at room temperature, filtrated and dried under nitrogen room temperature. Yield: 116.1 mg white crystalline solid, polymorph form B.

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100.2 mg polymorph form B are suspended in 2.0 ml EtOH in air. The white suspension is stirred in air over a weekend at room temperature, filtrated and then dried in air at room temperature. Yield: 94.2 mg of slightly yellow crystalline solid, polymorph form B. 119.1 mg of this slightly yellow crystalline solid, polymorph form B are suspended under nitrogen in 1.0 ml THF. The white suspension is stirred for about 20 hours at room temperature, filtrated and dried in air at room temperature. Yield: 114.5 mg of slightly yellow crystalline solid, polymorph form B.

126 mg of polymorph form B are suspended in 2.0 ml acetonitrile containing 2% by weight of water. The suspension is stirred for about 20 hours at room temperature under nitrogen atmosphere, filtrated and then drying under nitrogen. Yield: 116 mg of crystalline white solid, polymorph form B.

122 mg of polymorph form B are suspended in 2.0 ml ethyl acetate containing 2% by weight of water. The suspension is stirred at room temperature under nitrogen atmosphere for about 23 hours, filtrated and dried in air. Yield: 92 mg of crystalline white solid, polymorph form B.

366 mg of polymorph form B are stored in an open container under air at 75% relative humidity at 40° C. for 5 days. The solid is after this storage time at elevated temperature still polymorph form B.

## EXAMPLE A25

Preparation of Polymorph Form F of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A According to Example A1

102 mg of polymorph form A according to example A1 are suspended in 1.0 ml IPA. The suspension is stirred at room temperature under nitrogen atmosphere for about 19 hours, filtrated and dried in air. Yield: 102 mg of a crystalline white solid. Investigation of the obtained solid by powder X-ray diffraction and Raman spectroscopy reveals a crystalline form F. TG-FTIR: weight loss between 25-200° C. of 1.3% is attributed to water.

## EXAMPLE A26

Preparation of Polymorph Form F of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A According to Example A1

97 mg of polymorph form A according to example A1 are suspended in 2.0 ml IPA. The suspension is stirred at 10° C. for 22 hours, filtered and then dried under nitrogen at room temperature. Yield: 58 mg. The crystalline, white solid is polymorph form F, which shows the powder X-ray diffraction pattern as exhibited in table 4 and in FIG. 6.

TABLE 4

D-Spacings for form F		
Angle [°2θ]	d-spacings [Å]	Intensity (qualitative)
5.2	17.1	vs
7.3	12.1	w
10.3	8.6	w
12.7	7.0	w
13.6	6.5	w
13.9	6.4	w
15.0	5.92	w
15.5	5.72	w
17.4	5.11	w
18.0	4.92	m

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TABLE 4-continued

D-Spacings for form F		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
18.3	4.86	w
19.0	4.68	m
20.1	4.41	w
21.6	4.12	w
22.9	3.88	w
23.2	3.83	w
24.1	3.70	m
24.5	3.64	w
25.1	3.55	m
25.5	3.49	s
25.8	3.46	s
26.3	3.39	s
26.8	3.33	m
27.0	3.31	m
27.3	3.27	m
27.8	3.21	s
28.0	3.19	m
28.9	3.09	m
29.6	3.02	m
30.2	2.96	m
30.9	2.89	w
31.3	2.86	w
32.0	2.80	m
33.6	2.69	m

## EXAMPLE A27

Preparation of Polymorph Form J of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form E According to Example B8

250 mg of form E of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride are dissolved in 5.0 ml acetic acid and 1.0 ml water. To this solution 4.0 ml THF are added and the resulting suspension is slowly cooled to 5° C. Stirring is continued for about 16 hours before the suspension is filtered and obtained crystalline solid is dried under vacuum at ambient temperature. Yield: 179 mg of a crystalline white solid. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form J, which shows the powder X-ray diffraction pattern as exhibited in table 5 and in FIG. 10. TG-FTIR: weight loss between 25-200° C. of 0.6% is attributed to water.

TABLE 5

D-Spacing for form J		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
6.0	14.6	m
13.4	6.6	w
13.9	6.4	w
16.2	5.47	w
18.3	4.84	w
20.5	4.34	vw
21.2	4.20	vw
21.7	4.10	vw
24.3	3.67	w
25.2	3.54	w
27.1	3.29	vs
27.8	3.21	vs
30.3	2.95	w
31.5	2.84	vw
32.8	2.73	vw

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EXAMPLE A28

Preparation of Polymorph Form K of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

2.00 g of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride form B and 0.2 g of ascorbic acid are dissolved in 8.0 ml water. Subsequently, 40 ml acetic acid are added to this solution and then 30 ml of THF are slowly added to induce the crystallization. The resulting suspension is cooled to 0° C. and stirring is continued at 0° C. for about one hour before the solid is separated by filtration and washed with about 5 ml of ethanol of 0° C. The obtained crystalline solid is then again suspended in 30 ml ethanol at 0° C. resulting suspension is stirred at 0° C. for about 2 hours before the suspension is filtered and the obtained crystals are washed with 5 ml of ethanol of 0° C. The obtained crystals are dried at 30° C. under reduced pressure (8 mbar) for about 16 hours. Yield: 1.36 g of white crystalline solid. Investigation of the obtained solid by powder X-ray diffraction and Raman spectroscopy reveals a crystalline form K, which shows the powder X-ray diffraction pattern as exhibited in table 6 and in FIG. 11. TG-FTIR: weight loss between 25-200° C. of 0.6% which % is attributed to water.

TABLE 6

D-Spacing for form K		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
6.3	14.0	s
9.4	9.4	w
13.3	6.6	w
13.8	6.4	w
14.0	6.3	w
14.6	6.1	w
14.8	6.0	w
15.7	5.66	w
16.6	5.33	w
17.3	5.13	vw
18.8	4.73	m
19.1	4.64	m
19.8	4.48	w
20.5	4.32	vw
21.1	4.22	w
21.8	4.08	w
22.9	3.88	w
23.5	3.79	w
25.2	3.54	m
25.5	3.49	vs
26.3	3.39	m
26.8	3.33	vs
28.5	3.13	s
28.8	3.10	m
29.3	3.05	m
29.7	3.01	m
29.9	2.99	m
30.8	2.90	m

B) Preparation of Hydrate Forms of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

## EXAMPLE B1

Preparation of Hydrate Form C of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

116 mg of polymorph form B are suspended in 1.0 ml acetonitrile containing 50  $\mu$ l water. This suspension is stirred

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at room temperature for about 22 hours, filtrated and then dried in air at room temperature. Yield: 140 mg of a crystalline white solid, designated as form C. TG-FTIR shows a weight loss of 5.3% between 25 to 200° C., attributed to water and indicating a monohydrate. DSC: melting point near 94° C.,  $\Delta H \sim 31$  J/g. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form C, which shows the powder X-ray diffraction pattern as exhibited in table 7 and in FIG. 3.

TABLE 7

D-Spacing for form C		
Angle [°2 $\theta$ ]	d-spacings [Å]	Intensity (qualitative)
4.9	18.2	m
5.7	15.4	w
6.3	13.9	vs
8.5	10.4	w
9.2	9.6	w
9.4	9.4	vw
9.7	9.1	w
10.1	8.8	m
10.8	8.2	w
11.0	8.0	w
12.9	6.8	m
13.5	6.5	w
14.6	6.05	m
15.4	5.77	w
15.7	5.64	w
16.3	5.44	w
17.1	5.19	w
18.2	4.89	w
18.6	4.76	w
18.9	4.70	w
20.1	4.41	w
20.9	4.25	m
22.2	4.00	m
22.9	3.88	m
23.4	3.80	m
24.8	3.59	s
25.5	3.50	m
25.9	3.44	m
26.4	3.37	m
27.3	3.26	s
28.0	3.19	vs
28.1	3.17	s
28.7	3.11	m
29.2	3.06	m
29.6	3.02	m
30.1	2.97	vs
30.6	2.93	m
30.9	2.89	m
31.6	2.83	m
32.6	2.75	w
33.6	2.67	w
34.3	2.62	w
35.0	2.56	w
36.9	2.43	m

## EXAMPLE B2

Stability of Hydrate Form C of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

71 mg of hydrate form C according to example B1 are stored under 52% relative humidity and at room temperature for 17 days. Hydrate form C is retained.

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## EXAMPLE B3

Preparation of Hydrate Form D of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

A solution of 330 mg polymorph form B according to example A4 in 1.0 ml water is prepared. 600  $\mu$ l of this solution are added drop-wise to 10.0 ml 2-propanol at room temperature and stirred for about 2 hours. The precipitated solid is filtered off and dried at room temperature in air. Yield: 180 mg of a crystalline, white solid, designated as form D. TG-FTIR shows a weight loss of 4.8% between 25 to 200° C., attributed to water. Karl Fischer titration results in a water content of 6%. DSC: melting point near 153° C.,  $\Delta H \sim 111$  J/g. Investigation of the obtained solid by powder X-ray diffraction and Raman spectroscopy reveals a crystalline form D, which shows the powder X-ray diffraction pattern as exhibited in table 8 and in FIG. 4.

TABLE 8

D-Spacing for form D		
Angle [°2 $\theta$ ]	d-spacings [Å]	Intensity (qualitative)
9.1	9.8	vw
10.3	8.6	s
13.0	6.8	w
15.2	5.84	vw
16.0	5.56	m
17.8	4.99	m
18.1	4.90	vw
19.0	4.67	s
20.6	4.32	m
21.8	4.08	vw
22.6	3.93	vs
22.9	3.88	w
24.5	3.64	w
26.1	3.41	w
26.6	3.36	vw
27.4	3.25	w
28.2	3.17	m
29.3	3.05	s
30.4	2.94	w
30.6	2.92	w
31.0	2.88	m
31.4	2.85	w
31.9	2.80	m
32.1	2.79	m
33.1	2.71	vw
33.4	2.68	w
33.8	2.65	w
34.9	2.57	vw
35.6	2.52	vw
36.13	2.49	vw
37.58	2.39	vw
38.24	2.35	w
38.48	2.34	w
39.12	2.30	w
39.33	2.29	w

## EXAMPLE B4

Preparation of Hydrate Form D of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

246 mg of polymorph form B according to example A4 are dissolved in 4.0 ml IPA/H<sub>2</sub>O (4:1) at 40° C. 4.0 ml IPA are then added and the solution is cooled to 20° C. The formed suspension is stirred for about 20 hours at 20° C. The solid is filtered off and dried in air at room temperature for about 4

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hours. A comparison with the crystalline solid of example B3 reveals formation of hydrate form D.

## EXAMPLE B5

Preparation of Hydrate Form D of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

252 mg of polymorph form B according to example A4 are dissolved in 4.0 ml IPA/H<sub>2</sub>O (4:1) at 40° C. 4.0 ml IPA are added and the solution is slowly cooled to 5° C. At 25° C. 5 mg of seed crystals of form D are added. The temperature is changed to room temperature. The suspension is stirred for 40 hours, filtered and then dried in air for 5 hours at room temperature. A comparison with the crystalline solid of example B3 reveals formation of hydrate form D.

## EXAMPLE B6

Preparation of Hydrate Form D of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Hydrate Form C According to Example B1

700 mg of from hydrate form C according to example B1 are suspended in IPA/H<sub>2</sub>O (9:1). The suspension is stirred for 5 hours at room temperature, filtered and the solid dried in air at room temperature. Yield: 470 mg of white, crystalline solid, corresponding to hydrate form D.

## EXAMPLE B7

Treatment of Hydrate Form D of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
in isopropanol

105 mg of hydrate form D according to example B3 are suspended in 2.0 ml IPA. The suspension is stirred at room temperature for about 18 hours, filtered and the solid then dried in air at room temperature for about 4 hours. The obtained solid is the unchanged hydrate form D.

## EXAMPLE B8

Preparation of Hydrate Form E of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

489 mg of polymorph form B according to example A4 are dissolved in 1.0 ml water. The aqueous solution is added at 5° C. to 20 ml THF. The formed suspension is stirred for about 20 hours at 5° C., filtrated and dried under nitrogen at room temperature. Yield: 486 mg of a crystalline, pale yellow solid, designated as form E. TG-FTIR shows a weight loss of 10.8% between 25 to 200° C., attributed to water. Karl Fischer titration results in a water content of 11.0%, which suggests a dihydrate. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form E, which shows the powder X-ray diffraction pattern as exhibited in table 9 and in FIG. 5.

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TABLE 9

D-Spacing for form E			
Angle [°2θ]	d-spacings [Å]	Intensity (qualitative)	
5.7	15.4	s	
13.3	6.6	w	
13.7	6.5	w	
14.9	5.95	vw	
15.8	5.61	vw	
16.2	5.48	w	
16.9	5.24	w	
18.2	4.87	w	
19.7	4.50	vw	
20.8	4.27	w	
22.6	3.94	w	
23.6	3.78	w	
24.1	3.69	m	
24.8	3.60	w	
26.0	3.43	w	
26.8	3.33	s	
27.4	3.26	vs	
28.3	3.16	w	
29.0	3.08	m	
29.6	3.02	w	
29.9	2.98	w	
30.3	2.95	m	
30.7	2.91	w	
31.1	2.87	m	
32.0	2.79	w	
32.7	2.74	w	
33.2	2.69	w	
34.2	2.62	w	

## EXAMPLE B9

Preparation of Hydrate Form E of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

10 ml THF are cooled to 5° C. and then 400 µl of a concentrated aqueous solution containing about 160 mg polymorph form B according to example A4 is added drop-wise under stirring. The resulting suspension is stirred at 5° C. for about 2 hours at 5° C., then the precipitated solid is filtered off and dried in air at room temperature. Yield: 123.2 mg pale yellow crystalline solid, corresponding to hydrate form E.

## EXAMPLE B10

Preparation of Hydrate Form E of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

306 mg of polymorph form B according to example A4 are dissolved in 1.5 ml water. The water is evaporated from the aqueous solution under nitrogen at room temperature to dryness. The pale yellow crystalline residue corresponds to hydrate form E.

## EXAMPLE B11

Preparation of Hydrate Form E of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form A According to Example A1

71 mg of polymorph form A according to example A1 are stored in air under 52% relative humidity at room temperature for 17 days. The obtained pale yellow crystalline solid corresponds to hydrate form E. Hydrate form E is retained, when



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this solid is stored in air under 52% relative humidity at room temperature for 17 days.

## EXAMPLE B12

Preparation of Hydrate Form E of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

200 mg of polymorph form B according to example A4 are dissolved in 800  $\mu$ l water. 4.0 ml acetic acid and then 3.0 ml THF are added the solution. The suspension is stirred at 0° C. for 19 hours, the solid filtered off and dried in air at room temperature. Yield: 159 mg pale yellow crystalline solid corresponding to hydrate form E.

## EXAMPLE B13

Preparation of Hydrate Form H of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

250 mg of polymorph form B according to example A4 are dissolved in a mixture of 5.0 ml acetic acid and 1.0 ml water. To this solution are added 10 ml of THF as non-solvent. The obtained suspension is cooled to 0° C. and then stirred for 18 hours at 0° C. After addition of THF the void volume of the glass vial is purged with nitrogen and the cap is closed. The solid is filtered off and dried 24 hours room temperature under vacuum. Yield: 231 mg of a crystalline, pale yellow solid, designated as form H. TG-FTIR shows a weight loss of 6.5% between 25 to 200° C., attributed to water. Karl Fischer titration results in a water content of 6.34%. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form H, which shows the powder X-ray diffraction pattern as exhibited in table 10 and in FIG. 8.

TABLE 10

D-Spacing for form H		
Angle [°2 $\theta$ ]	d-spacings [Å]	Intensity (qualitative)
5.6	15.8	vs
8.6	10.3	vw
11.0	8.0	vw
13.4	6.6	vw
14.6	6.07	vw
18.5	4.81	vw
20.6	4.30	vw
23.0	3.87	w
24.7	3.60	w
27.3	3.27	w
27.8	3.21	m
28.5	3.13	vw
29.3	3.05	vw
30.2	2.96	w
31.0	2.89	w
31.8	2.82	vw
33.5	2.67	m

## EXAMPLE B14

Preparation of Hydrate Form O of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form F According to Example A26

About 50 mg of polymorph form F according to example A26 are placed on an powder X-ray diffraction sample holder of 0.8 mm thickness (TTK type, obtained from Anton Paar

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GmbH, Graz, Austria). The prepared sample holder is placed in the closed sample chamber of a Philips X'Pert powder X-ray diffractometer and the sample chamber is purged with nitrogen and partially saturated with water vapour to a resulting relative humidity of about 52%. After an exposure time of about 24 hour a powder X-ray diffraction pattern is recorded. Investigation of the obtained solid sample by powder X-ray diffraction reveals a crystalline form O, which shows the powder X-ray diffraction pattern as exhibited in table 11 and in FIG. 15.

TABLE 11

D-Spacing for form O		
Angle [°2 $\theta$ ]	d-spacings [Å]	Intensity (qualitative)
5.5	15.9	w
6.3	14.0	w
7.4	12.0	w
10.0	8.8	m
12.6	7.0	w
13.6	6.5	w
14.1	6.3	m
14.8	6.00	w
15.4	5.75	w
15.7	5.65	m
17.5	5.06	m
17.8	4.98	m
18.0	4.92	m
18.3	4.84	w
18.6	4.77	w
20.1	4.42	w
20.5	4.33	w
22.2	4.00	m
22.9	3.88	m
23.5	3.78	w
24.1	3.69	s
24.5	3.64	s
25.3	3.52	vs
25.5	3.49	s
25.8	3.46	s
26.1	3.42	s
26.8	3.32	m
27.3	3.27	m
27.6	3.23	s
28.0	3.18	s
28.3	3.15	vs
28.6	3.12	m
29.4	3.04	vs
30.3	2.95	m
31.8	2.81	s
32.9	2.72	m
33.6	2.67	m
34.3	2.61	m

C) Preparation of Solvate Forms of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride

## EXAMPLE C1

Preparation of Form G of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

245 mg of polymorph form B according to example A4 are suspended in 4.0 ml ethanol. 0.5 ml water are added and the mixture is heated to 70° C. to dissolve form B. The solution is cooled to 10° C. 2 ml of ethanol are added and the formed suspension is stirred for about 4 hours at 10° C. The solid is filtered off and dried for about 30 minutes under a slight flow of nitrogen at room temperature. Yield: 190 mg of crystalline white solid designated as form G. TG-FTIR shows a weight loss of 11.5% between 25 to 200° C., which is attributed to



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loss of ethanol and suggests an ethanol solvate. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form G, which shows the powder X-ray diffraction pattern as exhibited in table 12 and in FIG. 7.

TABLE 12

D-Spacing for form G		
Angle [°2θ]	d-spacings [Å]	Intensity (qualitative)
6.1	14.5	vs
8.1	10.9	w
9.0	9.8	w
12.7	7.0	w
14.1	6.3	w
15.4	5.74	w
16.9	5.24	vw
17.6	5.04	vw
18.5	4.79	w
20.1	4.41	w
22.1	4.02	w
23.0	3.86	w
23.6	3.77	w
24.1	3.69	w
24.6	3.63	m
25.0	3.57	m
25.5	3.49	m
26.2	3.41	m
27.3	3.26	m
28.1	3.17	m
29.0	3.07	m
30.1	2.97	m
30.3	2.95	m
31.2	2.87	w
34.3	2.61	w

## EXAMPLE C2

Preparation of Form G of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

200 mg of polymorph form B according to example A4 are dissolved in 400 µl water then precipitated with the addition of 10 ml ethanol. A precipitate is formed and the suspension is stirred for 17 hours at 0° C. The solid is filtered off and dried in air at room temperature for about 1 hour. Yield: 161 mg of crystalline white solid corresponding to ethanol solvate G according to example C1.

## EXAMPLE C3

Preparation of Form L of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Hydrate Form E According to Example B8

104 mg of hydrate form E according to example B8 are suspended in ethanol and the suspension is stirred at 4° C. for about 16 hours. The solid is filtered off and dried under nitrogen at room temperature. Yield: 100 mg of crystalline white solid designated as form L. TG-FTIR shows a weight loss of 9.1% between 25 to 200° C., which is attributed to ethanol and water. This weight loss suggests a mixed water/ethanol solvate. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form L, which shows the powder X-ray diffraction pattern as exhibited in table 13 and in FIG. 12.

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TABLE 13

D-Spacing for form L		
Angle [°2θ]	d-spacings [Å]	Intensity (qualitative)
6.3	14.1	vs
8.5	10.4	w
9.3	9.5	w
9.8	9.0	vw
12.9	6.9	w
13.6	6.5	w
14.4	6.1	w
15.4	5.75	w
15.8	5.61	w
17.5	5.08	w
18.9	4.71	w
23.1	3.86	w
23.5	3.78	w
25.7	3.46	m
26.5	3.36	m
29.2	3.06	w
30.8	2.90	w
31.8	2.82	w

## EXAMPLE C4

Preparation of Form L of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Form B According to Example A4

2.0 g of form B according to example A4 are dissolved in 3.0 ml of water. This solution is slowly added to 70 ml absolute ethanol (not denaturated) at room temperature. Approximately 300 mg of ascorbic acid are added to the aqueous solution and the void volume of the suspension is purged with nitrogen to prevent oxidation. The resulting suspension is cooled to 0° C. and stirred at this temperature for about three hours. Thereafter the suspension is filtered and the solid residue is washed with 6.0 g ethanol and dried for 18 hours at 35° C. under reduced pressure (8 mbar). Yield: 1.41 g. TG-FTIR shows a weight loss of 3.0% between 25 to 200° C., attributed to water. This results suggests that form L can exist either in form of an ethanol solvate, or in form of mixed ethanol solvate/hydrate, or as an non-solvated form containing as small amount of water. The solid residue comprises form L as shown by a comparison of powder X-ray diffraction pattern with that in example.

## EXAMPLE C5

Preparation of Form M of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

120 mg of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride according to example A4 are dissolved in 100 ml of absolute ethanol at 40° C. This solution is evaporated to dryness under a slight flow of nitrogen. The obtained crystalline white solid is designated as form M. TG-FTIR shows a weight loss of 9.1% between 25 to 200° C., attributed to ethanol and water, suggesting a mixed water/ethanol solvate. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form M, which shows the powder X-ray diffraction pattern as exhibited in table 14 and in FIG. 13.

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TABLE 14

D-Spacing for form M		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
4.7	18.9	s
13.9	6.4	m
14.6	6.06	w
15.7	5.66	w
16.8	5.28	w
19.7	4.50	w
21.0	4.23	w
27.7	3.22	vs

## EXAMPLE C6

Preparation of Form N of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from ethanol Solvate Form B According to Example  
A4

250 mg of form B according to example A4 are dissolved in 4.0 ml of a mixture of isopropanol and water (4:1). To this solution 4.0 ml of IPA are slowly added and the resulting suspension is cooled to 0° C. and stirred for about 18 hours at this temperature. The suspension is filtered and the solid residue washed with 4 ml of isopropanol at room temperature. The obtained crystalline material is then dried at 30° C. and reduced pressure (8 mbar) for about 18 hours. Yield: 150 mg. TG-FTIR shows a weight loss of 9.0% between 25 to 200° C., which is attributed to both isopropanol and water. This result suggests that form N can exist either in form of an isopropanol solvate, or in form of mixed isopropanol solvate/hydrate, or as an non-solvated form containing a small amount of water. Investigation by powder X-ray diffraction shows that the solid residue comprises form N, which shows the powder X-ray diffraction pattern as exhibited in table 15 and in FIG. 14.

TABLE 15

D-Spacing for form N		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
4.5	19.5	m
8.9	9.9	w
13.3	6.7	w
17.2	5.15	w
18.4	4.83	w
22.7	3.91	w
25.0	3.56	m
26.8	3.33	vs
28.3	3.15	w
30.9	2.89	w
31.9	2.81	w
35.1	2.56	w
38.2	2.36	w

## EXAMPLE C7

Preparation of Acetic Acid Solvate Form I of  
(6R)-L-erythro-tetrahydrobiopterin dihydrochloride  
from Polymorph Form B According to Example A4

252 mg of polymorph form B according to example A4 are dissolved at 40° C. in 4.0 ml acetic acid/water (4:1). 4.0 ml acetic are then added acid and the solution is cooled to 5° C. The resulting suspension is stirred for 66 hours. The solid is

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filtered off and dried in air for 5 hours at room temperature. Yield: 190 mg of crystalline white solid designated as form I. TG-FTIR reveals that form I contains about 12.7% by weight of acetic acid, which suggests an acetic acid solvate. Investigation of the obtained solid by powder X-ray diffraction reveals a crystalline form I, which shows the powder X-ray diffraction pattern as exhibited in table 16 and in FIG. 9.

TABLE 16

D-Spacing for form I		
Angle [ $^{\circ}$ 2 $\theta$ ]	d-spacings [ $\text{\AA}$ ]	Intensity (qualitative)
6.1	14.5	m
6.3	14.0	w
8.1	11.0	w
12.7	7.0	vw
12.9	6.9	vw
14.3	6.2	vw
16.7	5.30	w
18.5	4.79	w
20.0	4.44	w
20.7	4.29	w
21.2	4.20	vw
21.8	4.07	vw
22.1	4.02	w
23.2	3.84	w
23.4	3.80	w
24.2	3.67	vs
24.7	3.61	m
25.0	3.56	w
25.9	3.44	m
27.3	3.27	w
27.9	3.19	w
28.8	3.11	s
29.8	3.00	m
30.4	2.94	w
31.2	2.87	w
32.0	2.80	w

## Experimental:

Powder X-ray Diffraction (PXRD): PXRD is performed either on a Philips 1710 or on a Philips X'Pert powder X-ray diffractometer using  $\text{Cu}_{K\alpha}$  radiation. D-spacings are calculated from the 2 $\theta$  using the wavelength of the  $\text{Cu}_{K\alpha 1}$  radiation of 1.54060 Å. The X-ray tube was operated at a Voltage of 45 kV (or 40 kV with X'Pert Instrument), and a current of 45 mA (or 40 mA with X'Pert Instrument). A step size of 0.02°, and a counting time of 2.4 s per step is applied. Generally, 2 $\theta$  values are within an error of  $\pm 0.1$ -0.2°. The experimental error on the d-spacing values is therefore dependent on the peak location.

TG-FTIR: Thermogravimetric measurements are carried out with a Netzsch Thermo-Micro-balance TG 209 coupled to a Bruker FTIR Spectrometer Vector 22 (sample pans with a pinhole, N<sub>2</sub> atmosphere, heating rate 10 K/min).

Raman spectroscopy: FT-Raman spectra are recorded on a Bruker RFS 100 FT-Raman system with a near infrared Nd:YAG laser operating at 1064 nm and a liquid nitrogen-cooled germanium detector. For each sample, 64 scans with a resolution of 2  $\text{cm}^{-1}$  are accumulated. Generally, 300 mW laser power is used.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a characteristic X-ray powder diffraction pattern for form A

FIG. 2 is a characteristic X-ray powder diffraction pattern for form B

FIG. 3 is a characteristic X-ray powder diffraction pattern for form C

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FIG. 4 is a characteristic X-ray powder diffraction pattern for form D

FIG. 5 is a characteristic X-ray powder diffraction pattern for form E

FIG. 6 is a characteristic X-ray powder diffraction pattern for form F

FIG. 7 is a characteristic X-ray powder diffraction pattern for form G

FIG. 8 is a characteristic X-ray powder diffraction pattern for form H

FIG. 9 is a characteristic X-ray powder diffraction pattern for form I

FIG. 10 is a characteristic X-ray powder diffraction pattern for form J

FIG. 11 is a characteristic X-ray powder diffraction pattern for form K

FIG. 12 is a characteristic X-ray powder diffraction pattern for form L

FIG. 13 is a characteristic X-ray powder diffraction pattern for form M

FIG. 14 is a characteristic X-ray powder diffraction pattern for form N

FIG. 15 is a characteristic X-ray powder diffraction pattern for form O

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and, all parts and percentages are by weight, unless otherwise indicated.

The entire disclosure of all applications, patents and publications, cited herein and of corresponding U.S. Provisional Application Ser. No. 60/520,377, filed Nov. 17, 2003 is incorporated by reference herein.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

The invention claimed is:

1. A pharmaceutical tablet formulation comprising a purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, form B, which exhibits a characteristic X-ray powder diffraction pattern comprising characteristic peaks expressed in d-values (Å):  $8.7\pm0.1-0.2^\circ$  (vs),  $5.63\pm0.1-0.2^\circ$  (m),  $4.76\pm0.1-0.2^\circ$  (m),  $4.40\pm0.1-0.2^\circ$  (m),  $4.00\pm0.1-0.2^\circ$  (s),  $3.23\pm0.1-0.2^\circ$  (s) and  $3.11\pm0.1-0.2^\circ$  (vs); or which exhibits a characteristic X-ray powder diffraction pattern substantially as exhibited in FIG. 2; and polyvinylpyrrolidone as a pharmaceutically acceptable ingredient and/or dicalcium phosphate as an excipient, wherein the polyvinylpyrrolidone is a biologically degradable polymer which may be linear, branched or crosslinked.

2. A pharmaceutical tablet formulation comprising a purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, form B, which exhibits a characteristic X-ray powder diffraction pattern comprising characteristic peaks expressed in d-values (Å):  $8.7\pm0.1-0.2^\circ$  (vs),  $6.9\pm0.1-0.2^\circ$  (w),  $5.90\pm0.1-0.2^\circ$  (vw),  $5.63\pm0.1-0.2^\circ$

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(m),  $5.07\pm0.1-0.2^\circ$  (m),  $4.76\pm0.1-0.2^\circ$  (m),  $4.40\pm0.1-0.2^\circ$  (m),  $4.15\pm0.1-0.2^\circ$  (w),  $4.00\pm0.1-0.2^\circ$  (s),  $3.95\pm0.1-0.2^\circ$  (m),  $3.52\pm0.1-0.2^\circ$  (m),  $3.44\pm0.1-0.2^\circ$  (w),  $3.32\pm0.1-0.2^\circ$  (m),  $3.23\pm0.1-0.2^\circ$  (s),  $3.17\pm0.1-0.2^\circ$  (w),  $3.11\pm0.1-0.2^\circ$  (vs),  $3.06\pm0.1-0.2^\circ$  (w),  $2.99\pm0.1-0.2^\circ$  (w),  $2.96\pm0.1-0.2^\circ$  (w),  $2.94\pm0.1-0.2^\circ$  (m),  $2.87\pm0.1-0.2^\circ$  (w),  $2.84\pm0.1-0.2^\circ$  (s),  $2.82\pm0.1-0.2^\circ$  (m),  $2.69\pm0.1-0.2^\circ$  (w),  $2.59\pm0.1-0.2^\circ$  (w) and  $2.44\pm0.1-0.2^\circ$  (w); and polyvinylpyrrolidone as a pharmaceutically acceptable ingredient and/or dicalcium phosphate as an excipient, wherein the polyvinylpyrrolidone is a biologically degradable polymer which may be linear, branched or crosslinked.

3. A pharmaceutical tablet formulation comprising a purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride, form B, which exhibits a characteristic X-ray powder diffraction pattern substantially as exhibited in FIG. 2; and polyvinylpyrrolidone as a biologically degradable polymeric binder and/or dicalcium phosphate as an excipient.

4. The pharmaceutical tablet formulation according to claim 1, wherein the purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is of at least about 98% purity by high performance liquid chromatography.

5. A process for preparing a pharmaceutical tablet formulation according to claim 1 comprising

(a) providing a purified crystalline polymorph of form B of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride of at least about 98% purity by high performance liquid chromatography, and

(b) mixing the purified crystalline polymorph with polyvinylpyrrolidone and/or dicalcium phosphate.

6. A pharmaceutical tablet formulation prepared by the process of claim 5.

7. A pharmaceutical tablet formulation according to claim 1, further comprising folate alone or together with arginine.

8. A pharmaceutical tablet formulation according to claim 1, further comprising a lubricant, a vitamin and a sugar.

9. A pharmaceutical tablet formulation according to claim 2, further comprising a lubricant, a vitamin and a sugar.

10. A pharmaceutical tablet formulation according to claim 3, further comprising a lubricant, a vitamin and a sugar.

11. A pharmaceutical tablet formulation according to claim 1, wherein the polyvinylpyrrolidone is a biologically degradable crosslinked polymer.

12. A pharmaceutical tablet formulation according to claim 2, wherein the polyvinylpyrrolidone is a biologically degradable crosslinked polymer.

13. A pharmaceutical tablet formulation according to claim 3, wherein the polyvinylpyrrolidone is a biologically degradable crosslinked polymer.

14. The pharmaceutical tablet formulation according to claim 2, wherein the purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is of at least about 98% purity by high performance liquid chromatography.

15. The pharmaceutical tablet formulation according to claim 3, wherein the purified crystalline polymorph of (6R)-L-erythro-tetrahydrobiopterin dihydrochloride is of at least about 98% purity by high performance liquid chromatography.

16. The pharmaceutical tablet formulation according to claim 1, wherein the formulation comprises polyvinylpyrrolidone and dicalcium phosphate.

17. The pharmaceutical tablet formulation according to claim 2, wherein the formulation comprises polyvinylpyrrolidone and dicalcium phosphate.

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18. The pharmaceutical tablet formulation according to claim 3, wherein the formulation comprises polyvinylpyrrolidone and dicalcium phosphate.

19. The pharmaceutical tablet formulation according to claim 1, wherein form B exhibits a characteristic X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 5.63 (m), 4.76 (m), 4.40 (m), 4.00 (s), 3.23 (s) and 3.11 (vs).

20. The pharmaceutical tablet formulation according to claim 2, wherein form B exhibits a characteristic X-ray powder diffraction pattern with the following characteristic peaks expressed in d-values (Å): 8.7 (vs), 6.9 (w), 5.90 (vw), 5.63

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(m), 5.07 (m), 4.76 (m), 4.40 (m), 4.15 (w), 4.00 (s), 3.95 (m), 3.52 (m), 3.44 (w), 3.32 (m), 3.23 (s), 3.17 (w), 3.11 (vs), 3.06 (w), 2.99 (w), 2.96 (w), 2.94 (m), 2.87 (w), 2.84 (s), 2.82 (m), 2.69 (w), 2.59 (w) and 2.44 (w).

21. The pharmaceutical tablet formulation according to claim 19, wherein the formulation comprises polyvinylpyrrolidone and dicalcium phosphate.

22. The pharmaceutical tablet formulation according to claim 20, wherein the formulation comprises polyvinylpyrrolidone and dicalcium phosphate.

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